

Perceptions of Germany unveiled

Surveys have revealed the unpopularity of Germany as a place for young foreign scientists. In response, Germans need to improve conditions suffered by academics; foreigners need to think more realistically about the country and its inhabitants.

It is patronizing but apparently necessary to state that Germany is an exciting and fascinating place for scientists. Visit Max Planck Institute and you will be left in no doubt of the generous scale of funding received by heads of research groups, and the virtually complete autonomy they enjoy in the choice of topics on which to focus. Visit the more favoured laboratories in the east and you will encounter first-class researchers (many imported from the west) building new centres of excellence while grappling with post-reunification problems and opportunities. There are major facilities in plenty, and a fair crop of Nobel prize-winners. Long-term economic prospects are good, so Germany can be expected to go on dominating the league tables of research spending. It is boosting its spending on basic science.

So why do young scientists from other countries not like the place? New figures from the European Commission (EC) show it well down the list of desirable countries in which to spend a year or two doing research. More than 6,000 young scientists applied for fellowships to train in another European Union country under the EC's Human Capital and Mobility (HCM) programme, which ran from 1992 to 1994. Of these, 1,834 applied to work in Britain, 1,575 in France, but only 556 requested training in Germany. That sad situation persists in the HCM's successor programme Training and Mobility of Researchers (TMR). Barely 10 per cent of approved applications are to work in Germany.

Happily, the perceptions and prejudices that must underlie this abysmal response can be examined and confronted. A recent survey conducted by the German TMR coordinators among fellowship holders working in Germany throws some light on the issues. When asked what had most concerned them about Germany when considering where to train, they cited language, bureaucracy, hostility to foreigners, lack of accommodation and poor images of Germans. Those who took part in the survey, had, by definition, decided to work in Germany despite their misgivings. But the obstacles that others find insuperable need to be dealt with.

The language presents a problem — German is not widely studied in Europe and is undeniably difficult. The German TMR contact office points out that English is the international language of science and every scientist working in a laboratory will be able to speak it. It is true that German scientists are generally happy to practise their English, but long-term visitors wishing to participate in the day-to-day life of the laboratory will have to learn to speak German. The onus is on the visitor, but it is by no means an unrewarding one.

Bureaucracy is a different matter, at least at the universities, where researchers are confronted by extraordinary burdens of regulation. Again, the German contact office is keen to stress that fellows will not be hindered personally. Here there is a wider issue: both research and teaching at German universities are undermined by form-filling distractions, and change can come only with an urgently needed review of university management.

Whether something can be done about Germany's image is another matter. The country is burdened by history and is viewed in many places with suspicion and deep-rooted preju-

dice. Stereotypes of the German character are encountered all too often. Furthermore, elements of xenophobia in German society have received considerable publicity. But stereotypes evaporate in the fellowship of professional activity. Furthermore, younger Germans themselves treat history with caution — implications are discussed and confronted. And academics took to the streets in their thousands during 1992 to demonstrate against xenophobia: as in Italy and France, *Ausländerfeindlichkeit* is a serious but regionally contained problem. Such hostility is not likely to confront visitors — witness the hundreds of foreign scientists who work without problems every year in German laboratories, thanks to grants from the Deutscher Akademischer Austauschdienst and the Humboldt Foundation.

The internationalist morals of this tale are twofold. First, where historical burdens and international image are a problem, contacts with visitors can only be helpful. And second, with their own self-interest in mind, young European scientists should look twice at their perceptions, so as not to waste an opportunity to gain from Germany's scientific vigour. But an important goal for the country is to get university life into better shape. □

Bad day at Yucca

The delay in deciding on a site for permanent nuclear waste storage puts feeble politics ahead of decisive management.

HAZEL O'Leary, the US energy secretary, informed a Senate committee last week that permanent storage for spent nuclear fuel cannot begin at Yucca Mountain, Nevada, until 2015 at the earliest — a five-year delay necessitated, she says, by spending cuts. She added that President Bill Clinton would veto a bill proposed by Senator Larry Craig (Republican, Idaho) to establish an interim store at Yucca Mountain to hold the waste until then.

The Senate committee was rightly disconcerted to find that O'Leary has no satisfactory plan for dealing with the spent fuel in the interim. The energy department has a legal obligation after 1998 to take the spent fuel from nuclear power stations, where it is accruing at an alarming rate. But, according to O'Leary, Craig's proposed interim store at Yucca Mountain would upset the local inhabitants, who might then doubt the objectivity of a decision to put a permanent store there.

Add to this talk by Dan Dreyfus, her own nuclear waste manager, of a "very, very high probability" that the permanent facility can be built at Yucca Mountain, and one gains a clear picture of an administration fleeing from politically awkward decisions. Non-technical factors are at work — not least the Clinton administration's reluctance to do anything at all that might offend voters in nearby California.

Senator Bennett Johnston (Democrat, Louisiana) brands the position of his own party's administration on this issue "an outrage of astonishing dimensions". Such bluntness is justified. The nuclear waste issue is fraught with difficulty. It is nonetheless regrettable that the current administration so palpably lacks the courage to confront it. □



European proposal reopens debate over patenting of human genes

Munich. The European Commission is once again trying to clarify European patent law on biotechnological inventions by proposing a directive that would, among other things, explicitly allow patents on human genes isolated from the human body and on genetically altered animals and plants.

The proposed directive, which was published in Brussels last week, will be submitted for approval both to the Council of Ministers — the body representing the 15 member states of the European Union — and to the European Parliament. A previous draft, which had already been approved by the council, was unexpectedly rejected by the parliament earlier this year, largely because of opposition to the idea of patents on human genes (see *Nature* 374, 103; 1995).

In the new draft, the commission has responded to certain of parliament's concerns. The text has been clarified (for example, on the distinction between 'discoveries' and 'inventions' and the concept of 'farmers' privilege' which gives farmers the right to breed or propagate from patented animals and plants for their own purposes), and by specifically excluding patents on germ-line therapy techniques.

Compassion in World Farming, a British pressure group opposed to genetic engineering of animals, has welcomed a clause in the draft directive that specifies that genetically modified animals may be patented only if the value to humans of the modification outweighs any suffering to the animal.

But in general, members of the Green group in the European Parliament, as well

as other pressure groups concerned about the ethical aspects of genetic engineering, have already reacted critically to the new draft, saying that it remains "unacceptable" because it continues to allow patents on both genes and living organisms.

The new draft has been prepared under the direction of Mario Monti, the commissioner for the internal market and industrial affairs. He argues that the revised proposal "achieves the right balance between two equally essential requirements, [namely] promoting research and providing ethical protection".

Monti appears determined that, this time, the directive will be approved by the parliament (the embarrassment caused by the rejection of the first version was increased by the fact that it was the first time the parliament had used its recently increased powers to reject a directive approved by both the commission and European Union member states).

The new draft has therefore been drawn up after extensive consultation with both European parliamentarians and industrialists. It will also be discussed at a three-way public meeting between the parliament, commission and council on biotechnological issues, being held next month.

Monti: draft directive has 'the right balance'.

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Frank Spooner

Although some industry representatives doubt that the potential gains from a new directive will outweigh the public criticism the political debate is likely to generate, the commission has received backing from industrial lobby groups such as the Senior Advisory Group Biotechnology (SAGB) which represents around 30 biotechnology companies in Europe.

At the same time, environmentalist groups which successfully opposed the first draft of the directive argue that the commission's efforts to reopen the debate will provide them with a new opportunity for public expression of their concern.

One clause of the proposed directive likely to remain controversial seeks to clarify a passage in the European Patent Convention (EPC), which states that although animals and plants can be patented, animal and plant varieties cannot.

Those who oppose patents on living organisms argue that this should be interpreted to mean that, as new types of animals and plants can be considered as new collections of varieties, they are therefore unpatentable. Earlier this year, such an interpretation was accepted by an appeals board of the European Patent Office (EPO), which ruled that a patent granted to the Belgian company Plant Genetics Systems (PGS) on a procedure for producing pesticide-resistant plants could not cover the plants and seeds arising from the process (see *Nature* 374, 8; 1995).

An apparent corollary of this ruling — that plants as such are not patentable — led Paul Braendli, EPO's president, to refer the decision to the EPO's highest legal authority, the Enlarged Board of Appeals, which judges on apparent conflicts between appeal board rulings. Braendli argued that the ruling was in conflict with earlier decisions to grant patents on plants.

Last week, however, the Enlarged Board of Appeals announced that Braendli's request for its adjudication was "inadmissible", as the PGS appeal board's decision was not, in its view, directly contradictory to previous rulings.

Left with a precedent for disallowing patenting of plants — which opponents to the patent on the Harvard oncomouse are already arguing should cover animals as well — the EPO now finds itself back to the beginning in its struggle to interpret its own rules, laid down in the EPC. The directive proposed by the commission gives no clear-cut solution. But it does explicitly state that the exclusion of animal and plant varieties as such "does not prejudice the patentability of plants and animals".

Alison Abbott

AIDS patient given baboon bone marrow

San Francisco. A pioneering experiment involving the first-ever transplantation of bone marrow into a human being is proceeding well, according to researchers from the University of California at San Francisco and the University of Pittsburgh.

Last week, Jeff Getty, a 38-year-old AIDS patient, received baboon bone marrow, in the hope that a mixture of baboon and human marrow could help to reconstitute his damaged immune system. Baboons are not susceptible to HIV, and, if the experiment is successful, the chimaeric bone marrow will begin producing immune system cells resistant to the virus.

Several days after the transplant, Getty was reported to be doing well. But doctors said he would remain vulnerable to neutropenia — a decrease in the number of neutrophilic leukocytes in the blood — and opportunistic infections for several weeks.

Researchers should be able to measure how well the baboon cells have taken in about four weeks, but doubt they will see any immune reconstitution in under six months.

The protocol for the experiment had been given strict scrutiny by federal authorities, partly because of concern that the introduction of baboon cells into a human might trigger the development of new viruses. Suzanne Ildstad, a researcher involved in the experiment, said that monitoring for such organisms has already begun, with samples being collected weekly for examination by collaborators throughout the world.

Formal guidelines for xenotransplantation are being developed by the Food and Drug Administration and the Centers for Disease Control. Ildstad praised Getty's bravery and said many patients might ultimately benefit.

Sally Lehrman

NIH denies blame for radiation poisoning but tightens rules

Washington. The US National Institutes of Health (NIH) has denied any blame for a radiation poisoning incident on its Bethesda, Maryland, campus last June, arguing that it was effectively powerless to stop the "deliberate" contamination, and that confusion about dose estimates resulted from a lack of cooperation by the victim, Maryann Wenli Ma (see *Nature* 377, 568; 1995).

But the NIH has introduced tighter controls on the use of radioisotopes in the wake of the incident, with automatic suspensions from using or handling radioisotopes for 14–30 days for those who break them. The NIH disciplined 34 researchers last month under the new rules.

The NIH's denial was made in a formal response to the Nuclear Regulatory Commission (NRC), after Ma filed a petition asking the NRC to suspend the NIH's licence to handle radioisotopes. The NRC has not done so, but has insisted on a drastic tightening of controls on radioisotopes at NIH.

The NIH defends its response to the incident, in which the pregnant researcher ingested a large dose of the radioisotope phosphorus-32, as "immediate, thorough and entirely appropriate". But it says that Ma's "failure of full co-operation was a hindrance in the process", and that she repeatedly failed to provide urine samples.

Documents supporting the NIH's response claim that immediate action to dilute the radiation dose by prescribing sodium phosphate solution would have been inappropriate, as the P-32 would have been totally absorbed into the body by the time the incident was reported.

Debra Katz, one of Ma's lawyers, challenges NIH's contention that the correct application of the NRC rules could not have prevented what it concludes must have been a deliberate act. "Clearly, if you create a lax system, this kind of incident is far more likely to occur," she says. Katz adds: "We dispute that Dr Ma did anything other than what she was told to do". The incident is still being investigated by the Federal Bureau of Investigations.

Meanwhile, a report from the Institute of Medicine (IoM) calls for responsibility for regulation of radioisotopes in medicine and biomedical research to be taken away from the NRC and handed over to the states, under the supervision of the health department. NRC regulation is "costly and intrusive", says the IoM panel chaired by Charles Putnam of Duke University, North Carolina, adding that the commission should "immediately relax" its enforcement practices.

Colin Macilwain

Science champion perplexes allies with news of departure

Washington. Robert Walker, the Republican chairman of the Science Committee in the House of Representatives, surprised his political allies and critics alike last week with the unexpected announcement that he is to retire from Congress when his current term ends next November.

Since the Republican party won control of Congress a year ago, Walker has emerged as the most influential single figure in US science policy. He said last week that his departure was the result of "a personal decision" reached with his wife two weeks ago. "I really do believe this is the right time for me to go and do something else with my life," said Walker, who represents a district of the state of Pennsylvania.

Congressional staff are baffled at the announcement from a politician in the prime of life and career. Walker, who is 52, has been chairing the Science Committee for only a year, after two decades in political opposition. He is, in his own words, "in excellent political and physical health".

He denies one theory, that he is leaving because he is not going to be offered the chairmanship of the powerful Budget Committee, of which he is currently vice-chairman. "I never aspired to be chair of the Budget Committee," Walker said in the Science committee hearing room last Friday. "The only chair I ever aspired to was this one here."

As chairman of a committee with oversight responsibilities for most non-biomedical research in the United States, Walker has alienated parts of the research community by endorsing steep budget cuts in applied research programmes.

But he has been an enthusiastic supporter of basic science, and his links to leading Republicans — he has long been close to the speaker of the House, Newt Gingrich — has probably helped to protect both the National Science Foundation and physics programmes at the Department of Energy from budget cuts.

Jack Gibbons, science adviser to President Bill Clinton and one of Walker's main political adversaries, says that he considers Walker to have been "a very valued personal friend" who had been a strong advocate for science. "The research community will miss his support," adds Gibbons. (George Brown, now the senior Democrat on the Science Committee and its previous chairman, declined to comment on Walker's departure.)

Unlike many other retiring senators and congressmen, Walker had no worries about re-election, having received 70 per cent of the vote last year in his Pennsylvania district. With no children, and a congressional

district only a short drive from Washington, there has been no indication that the hectic pace of the Republican revolution was placing him under excessive strain. Furthermore, having spent his entire adult life in the US Congress — ten years on staff and 20 as a congressman — there is nowhere obvious for Walker to go.

But Walker justifies his decision by pointing out that no other congressman has served his district for more than 20 years. "As someone who came to the office promising myself that I would not spend the rest of my working life in the Congress, this is the right time to move on and in the process help keep a little bit of history intact," he says.

If the Republicans retain control of the House of Representatives next November, Walker is likely to be succeeded by James Sensenbrenner (Republican, Wisconsin). Sensenbrenner's main interest in the com-



Walker: denies that his retirement might mean abolition of the Science Committee.

mittee's work has always been in promoting the activities of the National Aeronautics and Space Administration (NASA) through the committee's space subcommittee, which he now chairs.

Democratic staff suggest that Walker's departure leaves the Science Committee open to abolition by Republicans keen to streamline Congress. Walker denies that this is likely to happen, or that his departure could mean the end of his pet scheme for linking various research-based agencies into a Department of Science. The logic for that is going to become "more pervasive" in future; "it didn't start with me, and it won't finish with me."

Sensenbrenner declined to comment last week on the issues he would like the Science Committee to tackle if he is offered the chairmanship. But he did confirm that, if such an offer was made, he would take it. "I've paid my dues, after all," he says, a reference to his 15 years on the committee, where he remains known primarily through his interest in NASA.

Colin Macilwain

Monterey woos biotech firms by relaxing field trial rules

San Francisco. Monterey County in California, the site of one of the earliest and most publicized controversies about agricultural biotechnology in the United States, has relaxed an eight-year-old law that has severely limited field experiments using genetically manipulated organisms (GMOs).

The law was one of the first attempts to place significant restrictions on the environmental release of GMOs, and is often cited as an example of public fear of the technology because of efforts to experiment with frost-resistant strawberries.

According to Richard Nutter, Agricultural Commissioner of Monterey County, the county's decision to soften its rules demonstrates a new acceptance and understanding of the technology. "Genetic engineering is becoming part of our culture," he says.

Monterey County is the salad bowl of the United States, producing \$2-billion worth of crops annually. Those who have been seeking reduced barriers to biotechnology have emphasized its potential for producing salt-tolerant and pest-resistant crops, as well as new types of agricultural products.

Until now, field trials of GMOs have required a special permit and approval from the county planning commission, whose review has included both traffic studies and an assessment of the local environmental impact. Studies within two miles of any occupied structure were prohibited.

These rules were approved about a decade ago, when Advanced Genetic Sciences, now known as DNA Plant Technology Inc., wanted to test a frost-protecting microorganism that it had developed on Monterey County strawberries.

Controversy over the proposal was particularly heated because the company had already tested the genetically altered organism illegally on the roof of its laboratories in Oakland. In addition, county officials had been unable to learn the location for the Monterey trial because of patent and trade secret restrictions.

The revised rules require field trials of GMOs to be carried out more than 100 feet away from an inhabited structure, providing that the trials meet state and federal safety regulations and that they have been approved by a committee made up of the agricultural commissioner, the county's head of environmental health and the director of the planning department.

The five-member county Board of Supervisors voted unanimously to make the change last month after only two members of the public had spoken against it, citing concern about pathogenic organisms in the

air, as well as the possible long-term consequences of the technology.

A nine-member planning commission also approved the ordinance. According to local officials, the effect of the previous ordinance put off all such research in the county, with not a single company applying for a permit to conduct field tests.

Sonya Hammond, director of the University of California Cooperative Extension for Monterey and Santa Cruz counties, called the law "a compromise". She said it resulted

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Ripe for release? Hostility to biotechnology has threatened to hit Monterey's agricultural economy.

from more than a year's effort by the extension, the county agricultural commissioner and others, to convince local policy-makers that the technology was both safe and beneficial for society. "You have to work with people to let them understand. Once they understand, they're more comfortable with it," she said.

Hammond worked with the University of California Systemwide Biotechnology Research and Extension Program to organize a conference on the technology last year for agricultural leaders and local officials. The programme focused on the science itself, the future direction of the technology and its potential benefits to Monterey County agriculture.

According to Nutter, county officials have recently felt that a failure to keep up with advances in biotechnology could rob the area of its agricultural base, as companies might choose to go to less hostile locations closer to their markets. The region also is suffering the economic fallout of the closure of the Fort Ord military base. The Monterey Bay Region Futures Network, an organization devoted to developing new economic alternatives, has pinpointed biotechnology as a potential growth opportunity.

The new ordinance is seen as the first step in a long-term plan that could eventually include a joint public-private partnership to develop a major agricultural research institute in the region. Agricultural biotechnology companies also have expressed interest in establishing sites there. **Sally Lehrman**

CERN urged to keep closer control over its staffing costs

Munich. Britain, Germany and Italy joined forces last week to fire a warning shot across the bows of both management and staff at CERN — the European Laboratory for Particle Physics — by voting against a new remuneration package, which gives staff a 0.7 per cent wage rise next year plus various additional benefits.

Although the three countries provide just over half of CERN's budget, the package was nevertheless approved at a meeting of CERN's council, as it required only a simple majority, and CERN's 16 other member states voted in favour (even though some are said to have been sympathetic to the arguments of the three dissenters).

Ken Pounds, chief executive of Britain's Particle Physics and Astronomy Research Council, and UK representative to the CERN council, says that by voting against the salary package, the three countries wanted to send a strong message of their concern that staff costs, which eat up half of CERN's total budget, must be strictly controlled.

But, he says, they were not unduly upset by the final outcome of the vote, as they had no desire to undermine the authority of the CERN management, headed by its director general, Christopher Llewellyn Smith, in its negotiations over conditions of service with staff members.

The three countries had pointed out that salaries at CERN are often considerably higher than those at comparable national laboratories in member states. But the CERN staff association counters by pointing out that staff salaries continue to lag behind those of scientists in comparable European organizations, such as the European Space Agency.

But various measures approved last week will have reassured the three countries who voted against the package at the council meeting. For example, there was agreement to recruit more short-term fellows and associate scientists at lower grades in the future, a measure that could be used to keep the total salary bill constant.

The laboratory's council also approved a new associate status for non-member states. This move is designed to encourage wider participation in CERN's SFr2.6-billion (US\$2.2-billion) Large Hadron Collider (LHC) project, whose construction was approved last year. Associate status will allow countries such as Japan, the United States and Russia to participate fully in the LHC project, with a voice in both the planning and running of the project, but without becoming full members of CERN, which wishes to restrict full membership to European states alone. **Alison Abbott**

SIPA-PRESS/Rex Features

Climate report cuts call for strong policies

London. The conclusions of a United Nations scientific document distilling the latest research on climate change appear to have been watered down to meet criticisms from both oil-exporting countries and the US government, which had suggested the document was too prescriptive in its proposals for future action.

The original 22-page draft document, based on the 3,600-page *Second Assessment Report* from all three working groups of the Intergovernmental Panel on Climate Change (IPCC), called for "strong policy measures" to limit and reduce greenhouse gas emissions.

But this phrase has been cut, as well as a whole paragraph setting out options through which countries can reduce greenhouse gas emissions. The new, 28-page final 'synthesis document' was approved last Friday at the end of a five-day meeting in Rome. Section eight of the document — called *The Road Forward* — its concluding segment, has been totally rewritten. Its final wording was strongly influenced by the United States.

Environmental groups say that they are satisfied with the overall thrust of the document. But they are unhappy at the removal of references to the need for strong policy measures. "The United States was unhelpful in this area," says Bill Hare,

climate policy adviser for Greenpeace.

Bob Watson, associate director of the US Office of Science and Technology Policy, and co-chair of IPCC Working Group Two, which deals with the impact of climate change and possible responses to it, says the changes were necessary. The original draft was much too long. "We made some suggestions to shorten it and make it less prescriptive," he says.

Watson says that an IPCC document always treads a fine line. "It will never be a smooth document when more than 100 countries are helping to write it. I can understand that some non-governmental organizations may not like the final version. But the draft version seemed to tell governments what to do. That is the role of the climate convention and its subsidiary bodies."

But Watson's view is challenged by Irving Mintzer, a senior research scholar at the Center for Global Change in Maryland. Mintzer, a lead author on Working Group Three — but not a member of the synthesis report's drafting team — denies that the team tried to "manipulate governments". The original section eight, he says, "pointed out what governments could do, but in a way that would advance national economic development priorities".

The new version does not mention nine possible "policies and instruments", contained in the original draft, through which countries could reduce greenhouse gas emissions. These included carbon taxes, deposit refund systems, subsidies, product bans and tradeable permits — where a country with high costs of reducing greenhouse gas emissions invests in emission-reducing measures in a country with lower reduction costs, but is credited for emission reductions in its own climate gas 'account'.

The original opening sentence in section eight stated that "future climate change will be determined primarily by the atmospheric concentration of carbon dioxide". This has also been deleted. A new sentence, inserted at the top of the second paragraph, states merely that "uncertainties remain" in the judgement of "what constitutes dangerous anthropogenic interference with the climate system".

A related paragraph that mentioned that carbon dioxide emissions needed to be stabilized at close to twice the pre-industrial levels "within a few decades" was also changed, with references to timescales taken out. Oil-producing countries have argued for longer and phased reduction of greenhouse gases to cushion the potential loss of oil export revenues.

Ehsan Masood

BSE results 'may quell panic', but caution still needed

London. Representatives of Britain's meat industry were expressing cautious optimism at the beginning of this week that new scientific data suggesting a lack of human susceptibility to bovine spongiform encephalopathy (BSE) — known as 'mad cow disease' — will help to stem a public panic that has seen beef sales fall by about 20 per cent in recent weeks.

"If it is true, it is obviously good news for industry," a spokesman for the Meat and Livestock Commission, the main industry trade body, said last Monday, 18 December. "From our point of view, anything which confirms what we have been telling the press for the past six weeks is very welcome."

The new data are contained in a paper published in today's *Nature* (see pages 779 and 761), and are based on work carried out by a team headed by John Collinge at St Mary's Hospital in London. An embargo on the results was lifted on Monday after reports on the research had appeared in the press the previous day.

The reported research shows that mice that had been genetically manipulated to react to the agent that causes Creutzfeldt-Jakob disease (CJD) in humans remained healthy when injected with the agent responsible for BSE. Collinge himself emphasizes that the findings only represent

the "first stage" in an overall experiment. The results reported so far cover only a comparison of initial survival rates between mice injected with CJD and BSE, while the full study, including evidence on the eventual mortality rates of the mice exposed to BSE — and which remain healthy — could take up to two years.

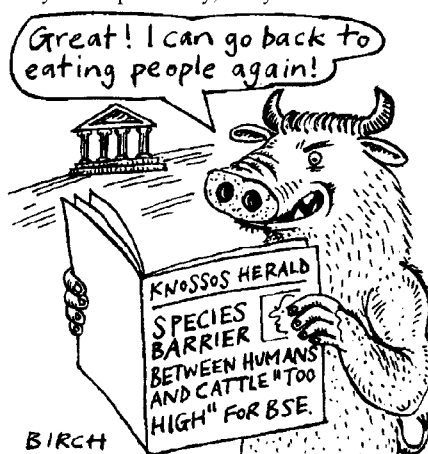
Indeed, there is some disagreement in the research community as to whether the initial data should have been published at this stage, without awaiting the results of the full study. "I think it is premature, both scientifically and politically," says John Bourne,

director of the Biotechnology and Biological Science Research Council's Institute for Animal Health. "None of this work has given us positive evidence [of the relationship between BSE and CJD] either way."

But Collinge himself claims that it would have been "unrealistic" to have awaited the full results of the study, given the significance of the results.

Indeed, some of those who have recently made public their own concern about a possible link between BSE and CJD say that they have been reassured by Collinge's results. Colin Blakemore, for example, Waynflete Professor of Physiology at the University of Oxford, who two weeks ago wrote an article advising people to give up eating beef "until the picture is clearer", described Collinge's work on Monday as "a very significant piece of evidence", and added "of course, I've changed my mind".

Blakemore says his earlier warning was based on the earlier lack of sufficient evidence to justify categorical statements from Stephen Dorrell, the health minister, that there was no evidence of a risk of contracting CJD from eating beef. "What I wanted was more caution — and more science," says Blakemore, adding that "[we] still need more results, and more epidemiological evidence; we must not relax our regulations". □



More on citation analysis

SIR — Further to the letter from Lewison *et al.*¹ the recently published Institute for Scientific Information (ISI) list of the most cited papers in the field of soil science² shows how misleading a mere citation count can be.

Out of the nine most cited soils articles, covering the years 1945–89, eight were to methods (804 to 368 citations per paper), mostly improvements on previous techniques, and only one to a lengthy review article of a multidisciplinary nature, which duly reached the list of 'citation classics' in *Current Contents*. The list does not include a single outstanding paper heralding significant advances in behaviour or process understanding of soil genesis or its more applied aspects.

Nobody would claim that in soil or other experimental sciences the methodology papers represent major scientific breakthroughs. These papers were merely fortunate that their methods had not been further improved and they therefore continue to be quoted. Soil science, like other Earth sciences, is both a basic science and regionally and spatially variable. Although its fundamental tenets and the methodology used are equal everywhere, significant advances in pedology are frequently of only regional importance and so may not be frequently cited.

In other fields, such as palaeontology, where precedence and the first report of an observation are important, the rules of citation also do not favour the most cited paper as representing the highest quality. In such cases, the numbers of citations, no matter how indexed, do not reflect quality or even usage, but are preliminary and incomplete guides to it. I have recently completed a study on paradigm shifts in soil science and doubt that all of the selected outstanding contributions would have made the list of most cited papers or books³.

There are many similar examples in other fields. No doubt insight in addition to rigorous number manipulation is after all equally important when evaluating a citation track record.

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SIR — I agree with Giovanni Motta⁴ that impact factor (IF) and citation index (CI) can be misleading. They often reflect not the quality of a piece of scientific work but the diffusion of the journal in which the work is published. But IF and CI values carry significant weight, and researchers compete fiercely to publish in journals with a high IF.

The dispute about how to judge scien-

tific work when appointing academics will continue for years to come. Unfortunately, in Italy, no account is taken of evaluation of academic disposition and scientific work in selecting candidates for Italian universities⁵. Indeed, it has been consistently shown that the most productive and respected candidates were failed in favour of candidates of lesser scientific merit.

Given the present practice, bearing the right surname, or having been a *portaborse* ('bag-carrier') to powerful professors, is more important than scientific prowess.

I agree that the selection process should not be based on totally mechanistic criteria. On the other hand, the Italian practice of leaving it totally in the hands of the panellists has resulted in the appointment to senior positions of candidates who have published hundreds of papers of dubious scientific value in non-refereed journals. The process of evaluation of a candidate's scientific work should not be regulated by tight laws, but neither should it be totally free. In countries without a damning past record of favouritism and nepotism, such as the United Kingdom, academic appointments are made 'in camera', and are seldom disputed.

As well as dedicating their time to bibliometric studies, researchers should probably investigate the heritability of the title of 'professor' in Italian academic families, which are well known and present in every university, especially in medical faculties. My conclusion, for the time being based on slightly more than anecdotal evidence, is that it must be an autosomal dominant gene or group of genes with an extremely high penetration.

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Spanish practices

SIR — Further to the letter from Vicente Rodilla (*Nature* **376**, 290; 1995) about hiring practices in Spanish universities, I would like to point out that, paradoxically, there appears to be a negative correlation at present between any objective and reasonable measurement of academic achievement in Spain and the probability of getting a permanent lectureship.

The main reason is that, whenever a vacancy occurs in a university department,

there is almost always a local candidate. If an outsider does not have an better academic record than the local candidate, there is little point in him or her applying for the post. Indeed, it is often the case that the local candidate is the only applicant, particularly if it appears that the selection has been decided in advance.

Furthermore, the most influential members of the five-person appointment committee in reaching a decision are the two members representing the university concerned, as they need the support only of one of the three other members for a particular candidate. These two will have little reason to select a candidate not favoured by the university, and will have little incentive to choose a candidate purely on merit, as their own situation is unlikely to be affected by their colleagues' achievements. Nor are any objective evaluations of university departments made public, so that the merits of a particular university cannot be assessed in advance by potential students.

Measures that might increase the teaching and research performance of Spanish universities include a public, external evaluation of the teaching and research activities of each department and the creation of a single applications process for all universities, open to all applicants in fair competition (in practice, there are currently quotas for non-local students). Both proposals would promote competition between universities. In addition, deans should be involved in all appointments, universities should have more salary grades, with internal promotion based on an external assessment of an individual's academic record, and appointment committees should include members from outside the university concerned.

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Still there

SIR — The 520 scientists who attended the 22nd International Cosmic Ray Conference in Dublin from 11 to 23 August 1991, which was hosted by the Dublin Institute for Advanced Studies, must have been astonished to read in your recent leading article (*Nature* **378**, 222; 1995) that "...after the Second World War...the Dublin Institute for Advanced Studies was active in a variety of modern fields, cosmic rays for example. But all trace of that endeavour had vanished by the mid-1960s..."

Cosmic ray physics is being actively pursued in the Dublin Institute for Advanced Studies as it has been since the establishment of the section in 1947.

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Mice and beef and brain diseases

James Hope

Can people contract neurodegenerative disease by eating beef products contaminated with bovine spongiform encephalopathy? Experiments with transgenic mice give cause to think that the answer may be 'no'.

ON page 779 of this issue¹, a group led by John Collinge describe their failure, to date, to transmit the infectious agent thought to be responsible for bovine spongiform encephalopathy (BSE) to mice that have been genetically engineered to carry the human prion protein. Prion proteins are found in the brain and, when converted into an enzyme-resistant form, result in a variety of neurodegenerative diseases (see box). The object of the experiments is to find out whether BSE can cross the species barrier to humans, and manifest as Creutzfeldt-Jakob disease (CJD) in people who have eaten infected beef. Although these studies are not yet complete, this news should provide some comfort for consumers.

Ever since BSE was diagnosed as a prion disease²⁻⁴, similar to CJD of man, speculation has been rife that people might develop a CJD-like illness by eating

beef and other cattle products. The low incidence and long incubation periods of these diseases — several decades in some cases — mean it could take many years before CJD surveillance reveals whether or not this is happening, so there is a pressing need to develop quicker ways of assessing the risk of transmission of prions from cattle to man. Obviously you can't do this by injecting BSE directly into people, and an alternative way of tackling the problem is to use mice expressing the human prion protein (HuPrP) for transmission studies⁵.

The prion is a synonym for the infectious agent of BSE or CJD, but the prion protein (PrP^C) itself is a harmless glycoprotein, of relative molecular mass 35,000, that is normally found on the surface membrane of cells in brain and other tissues. A key process in the development of CJD and related diseases is the conver-

sion of PrP^C to a protease-resistant isoform (PrP^{Sc}). PrP^{Sc} is enriched in preparations of infectious particles and, either by itself or in association with other molecules, is regarded by many as the infectious agent or prion. PrP^C is obligatory for the replication of the infectious agent, production of PrP^{Sc} and the development of disease in transgenic mice⁶, although the prion model is not universally accepted because of its failure adequately to account for strain variation⁷.

One version of the prion hypothesis holds that the formation of new PrP^{Sc} results from the interaction between pre-existing PrP^{Sc} and PrP^C, with the efficiency and rate of this process — and hence the timing and tempo of disease — determined in part by the compatibility in the structures of the host (PrP^C) and agent (PrP^{Sc}) proteins. This view is supported by the transmission of scrapie isolates to transgenic mice, where the course of disease is accelerated by matching the PrP type of the donor (PrP^{Sc}) with that of the recipient (PrP^C)⁸. So a HuPrP transgenic mouse, that is, one expressing human PrP, may be a more sensitive animal for assessing the potential of prions in human and cattle tissues and body fluids to transmit disease to humans. This reasoning is borne out by some of the results published in this issue.

Collinge *et al.* have inoculated non-mutant (wild-type) mice and various HuPrP transgenic mice with tissue homogenates from the brain of a CJD patient or from a pool of brains from BSE-affected cows (see figure). The survival times of HuPrP transgenic mice inoculated with CJD agent (250–300 days) were significantly shorter than those of wild-type mice (*Prn-p*^{+/+}, represented here as *HuPrP*^{0/0}:*MoPrP*^{+/+}), which lived for 480 days or more. The incubation periods in transgenic mice expressing HuPrP against a background of mouse PrP were inversely related to the amount of HuPrP^C expressed in the brain — 244 days in high expressors compared to 297 days in low expressors — and further decreased in mice expressing only HuPrP (*HuPrP*^{+/0} or *HuPrP*^{+/+}:*MoPrP*^{0/0}) (196–212 days). Inoculation of brain homogenate from cases in *HuPrP*^{+/0} transgenic mice into more *HuPrP*^{+/0} mice or wild-type mice gave similar differences (213 days compared to more than 420 days, respectively), indicating that the expression of HuPrP may lower the barrier to transmission of CJD

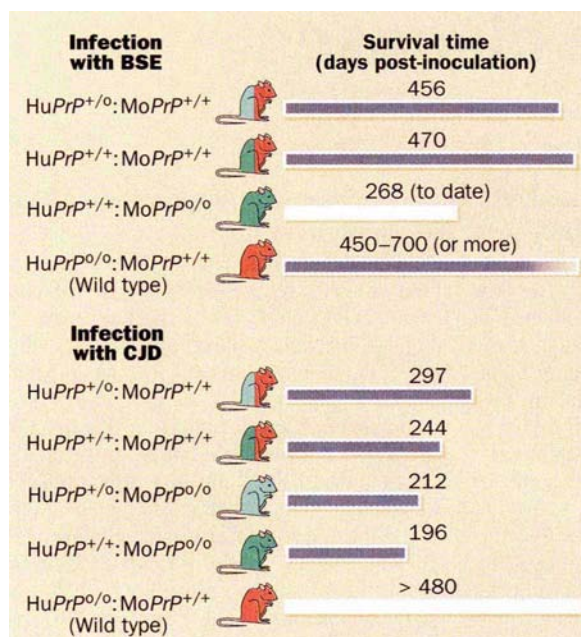
A short history of prions

SCRAPIE, Creutzfeldt-Jakob disease (CJD) and bovine spongiform encephalopathy (BSE) are neurodegenerative diseases of sheep, humans and cattle, respectively. They are transmissible by feeding or inoculation and have incubation periods varying from a few months to several years depending, primarily, on dose, route of inoculation, inoculum strain and the genetics of the infected species. In the 1950s, infectivity from extracts of scrapie-affected sheep was shown to pass through filters with pores small enough to retain all but viruses. The infectious factor had some of the attributes of a virus, such as strain variation, but what sets scrapie and related encephalopathies apart from other diseases is the relative resistance of this virus-like activity to inactivation by procedures, such as boiling or exposure to ionizing and ultraviolet radiation, that kill viruses.

In 1967, Griffith¹⁰ suggested that, unlike a virus, a nucleic-acid template might not be required for replication of the scrapie phenotypes and that a protein was the sole or an integral part of the infectious particle. In 1982, Prusiner and colleagues¹¹ discovered a protein that fitted the role of Griffith's putative molecule and dubbed it the prion protein (PrP), and re-named the

infectious unit a prion (a jumbling of 'proin' from 'proteinaceous infectious particle'). In the prion model, the infectious agent is a modified form of a host-encoded glycoprotein (PrP^C); the conversion of PrP^C to the modified form PrP^{Sc} is proposed to occur either by direct interaction of PrP^C with PrP^{Sc} ('the infectious agent'), or in the case of spontaneously arising or genetically transmitted disease (for example, CJD or natural scrapie) by a rare, chance event that sparks off the self-replicating, catalytic conversion of PrP^C to PrP^{Sc}. In a series of elegant experiments, this conversion has been replicated in a test tube^{12,13}, although it remains to be shown that this biochemical change is equivalent to *de novo* synthesis of infectious particles.

A great many of the physicochemical and epidemiological features of these diseases can be explained by this simple one-component model. But, as yet, the mechanism by which different strains of agent have different effects in the same strain of mouse (which possesses a single PrP^C amino-acid sequence) is more difficult to envisage, and some research groups continue to maintain that a second molecule, not PrP^C, determines the strain of the infectious particle. J. H.



Incubation periods (grey bars) or survival times (open bars) in transgenic or wild-type mice following injection with BSE or CJD brain homogenates. HuPrP denotes a human prion protein gene array — 30–50 copies of the gene integrated into chromosomal DNA, with heterozygotes (HuPrP^{+/+}; pale green) producing twice the amount of human PrP and homozygotes (HuPrP^{+/+}; dark green) 4–8 times that found in the same weight of human brain; similarly, MoPrP^{+/+} is the wild-type mouse genotype (red) with two copies of the mouse PrP gene per diploid cell. Different wild-type mouse strains produced a wide range of incubation periods when inoculated with BSE (ref. 4) or CJD (ref. 14).

to mice by intracerebral inoculation. This is in accord with a simple model of PrP^C–PrP^{Sc} interaction — or one in which PrP^C acts as a receptor for a virus. But, as the authors recognize, it is based on transmission from a single case of CJD to mice expressing only one of the common forms of HuPrP (one with valine at codon 129)⁹.

Collinge *et al.* then went on to see whether or not these HuPrP transgenic mice could be used to find out if there is a species barrier limiting transmission of BSE to humans. In contrast to the results of the studies on CJD transmission, the authors found that following inoculation with BSE agent the survival times of transgenic mice expressing both mouse and human PrP were not significantly different from those of the parent strains from which the transgenic mice had been produced (450–520 days). This is well within

the range of incubation periods reported some years ago⁴ for the transmission of several BSE isolates to a panel of conventional mice with 100 per cent susceptibility, and suggests that the presence of HuPrP does not significantly reduce the incubation period following injection with BSE.

Furthermore, only MoPrP^{Sc}, not HuPrP^{Sc}, was identified biochemically in brain extracts from these mice, although, as the authors acknowledge, this is not a sensitive indicator of low levels of infectivity. An important part of the design of this experiment to rule out the production of HuPrP^{Sc}-based infectivity is to inoculate HuPrP mice with brain extracts from these HuPrP/MoPrP mice. This experiment is underway, but as yet incomplete, and it will be another two years before replication of human infectivity in these mice can be discounted.

The HuPrP transgenic mice lacking MoPrP, which show shorter incubation

periods following CJD challenge than the mixed HuPrP/MoPrP mice from which they were derived, have also been inoculated with BSE. To date, 268 days after

they were injected, these mice have not developed disease. Although this survival time is already 63 days longer than that for the CJD isolate injected into mice of this genotype, it should not be confused with a decreased susceptibility to infection — a short incubation period is not equivalent to high susceptibility and depends on the strain and amount of prions injected, neither of which can be assumed to be equivalent in these CJD and BSE isolates. Transmission of BSE to some wild-type strains of mice has resulted in neurological disease in all recipients after incubation periods of 600–700 days⁴ (100 per cent susceptibility), so it would be premature to say that these HuPrP transgenic mice are not susceptible to BSE.

Finally, what would be the implications for human health if the transgenic mice were eventually to produce human PrP^{Sc} and die of BSE? This is a very difficult question but obviously you can't switch a mouse into a human being by changing a single gene (a caveat that will also apply if the mice don't get sick). Finding animal analogues of human infectious diseases is a common problem and the HuPrP mouse is an encouraging development. But we need much more detailed knowledge of the comparative pathogenesis of neurodegenerative diseases in humans and transgenic mice before an answer emerges — unsatisfying as that will be to consumers and producers of beef. □

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BREAST CANCER GENES

Further enigmatic variations

Kevin Davies

AT about the same time last year as scientists finally got their hands on the first gene for hereditary breast and ovarian cancer, *BRCA1* (ref. 1), researchers also announced² that a second familial breast cancer gene, *BRCA2*, had been assigned to the short arm of chromosome 13. So no sooner had one high-profile genetic quest been concluded than another one was launched. Some 15 months later, that race, too, is over, for on page 789 of this issue³ Michael Stratton and 37 colleagues present convincing evidence that *BRCA2* has been identified.

Breast cancer takes a large toll of women throughout the Western world. In the United States alone, there are 182,000 new cases each year, and 46,000 women die of the disease. Debate continues as to the likely epidemiological causes — dietary fat, pesticides and electromagnetic radiation are just a few of the suspected

risk factors — but in about 5% of cases the disease is hereditary. In these families, patients develop breast (or ovarian) cancer relatively early, before menopause, presumably because of the absence of one copy of a tumour-suppressor gene such as *BRCA1* or *BRCA2* in the germline.

After *BRCA1* was mapped to chromosome 17 in 1990, there were high hopes that its eventual isolation would generate profound insights into the cellular basis of breast (and ovarian) cancer. So far, the results have been disappointing: the protein encoded by *BRCA1* is large, with only a DNA-binding zinc-finger motif near the amino terminus offering any hints as to its function. Furthermore, although more than 50 separate germline mutations in *BRCA1* have been identified in hereditary cases of cancer⁴, alterations of the gene in somatic cells have been described in only a few sporadic ovarian (but not breast)

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tumours^{5,6}. There has been a recent suggestion that the *BRCA1* protein is mis-localized in the cytoplasm of sporadic breast tumour cells⁷, but confirmation and insight into the mechanism of such an effect require further work.

Although *BRCA1* accounts for breast and ovarian cancer in more than 75% of families with a genetic predisposition to the diseases⁸, mutations in *BRCA2* probably cause at least one-third of cases of familial breast (but not ovarian) cancer; these families also suffer from a higher incidence of male breast cancer. In other words, *BRCA2* is an important 'breast cancer' gene in its own right. Over the past 12 months, many groups have been analysing more families and new polymorphic markers in the hope of identifying key recombinant individuals that might pinpoint the location of *BRCA2*. In this way, Wooster *et al.*³ narrowed the interval containing *BRCA2* to about 600 kilobases.

To reduce the gap still further, Wooster *et al.* decided to concentrate on a 300-kilobase section of their critical region that had been shown to be deleted homozygously in a pancreatic tumour⁹. Although at best this was a tenuous link, the deletion was too intriguing to be dismissed as simple coincidence. Using exon trapping and direct selection techniques, the group then isolated part of a gene from a genomic-DNA contig spanning this interval, and proceeded to screen DNA from nearly 50 patients in search of mutations. The first mutation they encountered — a 6-basepair deletion removing a splice site and producing a premature stop codon — was encouraging.

Wooster *et al.* went on to identify five other mutations of a similar nature, all of which are probably capable of disrupting the natural function (whatever that may be) of the protein encoded by *BRCA2*. Interestingly, three mutations in male breast cancer patients are among the collection. As for the putative function of *BRCA2* protein, there is little to say at this juncture. Only weak homologies to known proteins have been found, but among them is a region of similarity to *BRCA1* (not, however, in a portion of known function). Who knows, though, whether more substantive similarities (such as another zinc finger) will be uncovered when the full sequence is completed shortly? □

Kevin Davies is editor of *Nature Genetics*.

Light from tungsten on core construction

David J. Stevenson

THE Earth's iron core is thought to be almost as old as the Earth itself (approaching 4.6 billion years) but there are different opinions as to how quickly and in what manner it formed. Core formation times as short as 10 million years or longer than the time to accrete the Earth (around 100 Myr) have been suggested. This question is very relevant to how planets are assembled and how all the terrestrial reservoirs (ocean, atmosphere and mantle as well as core) are established, and it can only be addressed by radioisotopic dating methods. In this regard, a promising development is hafnium-tungsten chronometry, and on page 771 of this issue, Lee and Halliday¹ use this approach to infer that the core formation took longer than about 60 million years. Moreover, their interpretation of new meteoritic data implies that there must have been an unexpectedly high level of ¹⁸²Hf in the early Solar System.

According to current ideas and numerical models of terrestrial planet formation², the Earth formed over a period of tens of millions of years and most of the mass arrived in bodies that were considerably larger than the Earth's Moon, perhaps including bodies larger than Mars. Although a timescale approaching 100 Myr is often quoted for the Earth's formation, a large fraction of the Earth's mass (say 60 per cent) can aggregate in a timescale as short as ten million years. The 'timescale' of Earth formation is thus a fuzzy concept, partly because the process is stochastic and partly because it depends on how you define it. (The Earth is still growing in mass, through impacts with comets and asteroids, albeit very slowly.)

Segregation

A simple view of core formation attributes the process to impact-induced melting within the growing planet³. This partial or even total melting of initially well-mixed materials (metallic iron, oxides and silicates) allows an immiscible iron-rich liquid to migrate and aggregate into large bodies which then descend under the action of gravity. Physicists and fluid mechanicians will recognize this as a Rayleigh-Taylor instability. Calculations suggest that the time for a large blob of iron to descend to the core is short compared with the timescale of planet formation, so the real issue is the time it takes to develop the conditions needed for melting and segregation or the rate at which iron is delivered to the growing planet, rather than the time for iron to

sink. If there were a single event that dominated core formation then one could define sharply the time of last chemical equilibration between the core-forming melt and the silicates and oxides (the proto-mantle), and perhaps be able to use hafnium-tungsten chronometry to determine this date.

Chronometry

Let us think through how the chronometry would work in a particularly simple case, even though the real world may not be so obliging. We imagine forming the Solar System from the collapse of a homogeneous interstellar cloud of gas and dust that contained within it many short-lived radioisotopes arising from the incorporation of nucleosynthetic material expelled from neighbouring stars. In particular it contains ¹⁸²Hf which decays with a half-life of 9 Myr to ¹⁸²W. Hafnium and tungsten are incorporated into small solid bodies, some of which then differentiate to form metallic cores very quickly (and most probably because of the heating produced by some even shorter-lived radioisotopes, particularly ²⁶Al).

During this core formation event in small bodies, most of the tungsten goes into the core because it is 'iron-loving' (siderophilic), but most of the hafnium stays in the silicate portion (the mantle). These cores would contain the interstellar cloud abundance of ¹⁸²W, uncontaminated by the ¹⁸²W that comes from ¹⁸²Hf decay, because the latter would form later and mainly in the mantle. Some of these small bodies are subsequently disrupted by collisions and the debris from their cores can include iron meteorites that end up on the Earth. The first tungsten isotopic measurements of such an iron meteorite were made by Harper and colleagues⁴, and the results now obtained by Lee and Halliday confirm their work. As always in such work, the results are expressed as a ratio of isotopes, ¹⁸²W/¹⁸⁴W, to cancel out any chemical effects that shift absolute abundances by large amounts yet leave isotopic ratios unchanged. (The other isotope, ¹⁸⁴W, has no important radioactive precursor and both tungsten isotopes are stable.)

Now suppose that we could get hold of undifferentiated bodies which contain within them both the initial ¹⁸²W and whatever ¹⁸²W comes from hafnium decay. Carbonaceous chondrites are presumably from such bodies, and Lee and Halliday have analysed both Allende and Murchison, prime examples of these primitive, undifferentiated meteorites. Their results

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(presumably representative of the entire meteorite and the body from which they came) show a higher $^{182}\text{W}/^{184}\text{W}$ than in the iron meteorites. The implication is that substantial amounts of live ^{182}Hf were present at the time of formation of the iron cores from which the iron meteorites were derived. Assuming that this core-forming event pre-dates significant ^{182}Hf decay, the initial value of $^{182}\text{Hf}/^{180}\text{Hf}$ consistent with their results is around 2×10^{-4} , about an order of magnitude higher than previous estimates or the predictions of current nucleosynthetic models⁵.

We come now to the Earth, the silicate portion of which is found by Lee and Halliday to have a value of $^{182}\text{W}/^{184}\text{W}$ that is indistinguishable from Allende or Murchison. This can be seen to be a most important result. Suppose that the Earth's core had formed as a single event and before most of the decay of ^{182}Hf had taken place. Then most of the tungsten would have been stripped from the silicate portion and whatever was left would have been augmented by the decay of ^{182}Hf . If the stripping process was strong then the mantle $^{182}\text{W}/^{184}\text{W}$ could be greatly amplified by subsequent ^{182}W production, and one could end up with a $^{182}\text{W}/^{184}\text{W}$ that is much higher than the initial Solar System value, the difference being much greater than the difference between the tungsten isotope ratios for the iron meteorite and the carbonaceous chondrites.

The absence of such a potentially large signal in the silicate Earth suggests that core formation occurred many half lives of ^{182}Hf after iron core formation in meteorite parent bodies. Thus Lee and Halliday are able to infer a timing of around 60 Myr or more for the 'event' that formed the Earth's core. They also find a similar isotope ratio for a lunar mare basalt, suggesting a lunar formation timing similar to core formation timing, compatible with some ideas of giant impact origin for the Moon⁶.

Lee and Halliday's is an impressive technical and scientific accomplishment. But does it tell us what we want to know? Even if the results are completely confirmed, the strength of their conclusions lies in the credibility of the assumptions and the models employed. The Earth's core formation might have been preceded by core-forming events in the large planetesimals that then accreted onto the Earth. If the cores of these projectiles were only partially disrupted during impact, then the chronometer would date the earlier core-forming events and not the Earth's core aggregation (core 'mergings'). The multistage character of planet accumulation makes it difficult to define what is meant by the timing of core formation and even more difficult to measure it.

There is also the issue of whether the

terrestrial tungsten reservoir of the mantle could have been augmented after core formation by late infalling material (which might well have a $^{182}\text{W}/^{184}\text{W}$ like that of Allende and thus be like what we now see in the Earth's mantle). Lee and Halliday make the point that even if there is dilution of this kind, the value of $^{182}\text{W}/^{184}\text{W}$ is so extreme before dilution that it would still be apparent (albeit attenuated) in the Earth standards they have so carefully measured. Although their argument is qualitatively sound, one wonders whether a detailed model with overlapping processes and imperfect core formation would yield such a firm conclusion for all plausible model parameters.

Many people may be comfortable with the result obtained by Lee and Halliday, because it conforms roughly with the timescale commonly associated with Earth formation. But it is unclear whether they should be comfortable. Of course, there are many non-isotopic considerations involved in core-mantle partitioning that might help constrain the physical circumstances of core formation, and these are currently receiving much attention. One would like to see confirming evidence for the high required ^{182}Hf , and a

striking confirmation would be the observation of very high values of $^{182}\text{W}/^{184}\text{W}$ in those meteorites that might be complementary to the 'irons' (in other words, meteorites from the mantles of the small bodies from whose cores the iron meteorites are derived). The meteorite classes known as pallasites and eucrites are good candidates. Perhaps the most immediate challenge posed by Lee and Halliday is to those who seek to explain early Solar System abundances through nucleosynthetic and stellar modelling. Why so much ^{182}Hf ? □

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DINOSAURS

Brooding with the best

David B. Weishampel

OVER the past two decades it has become plain just how important dinosaurs were in the ecology of the Mesozoic Era, some 65 to 245 million years ago, and in the evolutionary history of modern birds. More recently, discoveries of not only 'subadults', but also of well-preserved youngsters, hatchlings and even embryos, have enriched our understanding of dinosaur growth and development¹. In turn, these specimens provide a window onto such ephemeral aspects of organismal biology as dinosaurian reproduction and life histories, none of which had been

contemplated previously. On page 774 of this issue², Norell and colleagues go beyond eggs and neonatal specimens to provide astonishing and incontrovertible evidence of avian brooding behaviour in a non-avian dinosaur.

The discovery of dinosaur eggs and juveniles is not new. The first dinosaur eggs were found in the French Pyrenees, but the earliest discoveries to capture the public's attention were made in the Gobi Desert by the Central Asiatic expedition mounted by the American Museum of Natural History during the 1920s. The expedition collected several clutches of eggs, as well as juvenile, subadult and adult skeletons of a small ceratopsian dinosaur — named *Protoceratops* — which was regarded as having produced these eggs. The possibility of a similar windfall of fossils from the Late Cretaceous (65–97 million years ago) was to bring successive expeditions to southern Mongolia for the next three decades³: the joint Soviet–Mongolian expeditions of the 1950s and 1970s; the Polish–Mongolian expeditions of the 1960s and early 1970s; and, most recently, the expeditions of the Hayashibara Museum of Natural Sciences and Mongolian Academy of Sciences, and of the Mongolian Academy of Sciences and the American Museum of Natural

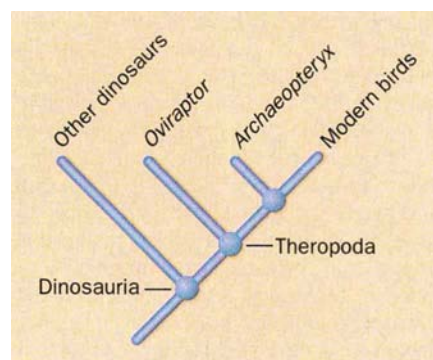


FIG. 1 Simplified cladogram of Dinosauria, indicating the close evolutionary relationship of *Oviraptor* to *Archaeopteryx* and modern birds.



FIG. 2 Head of *Oviraptor*, showing the projecting cassock, beak and two peg-like projections in the centre of the palate; skull length is about 20 cm. Reconstruction by Greg Paul.

History. This last project has been exceptionally successful, particularly at Ukhaa Tolgod in south-central Gobi, where new kinds of dinosaurs and spectacular preservation — including eggs containing embryos — have been found⁴⁻⁶.

Among the most abundantly preserved dinosaurs at Ukhaa Tolgod are oviraptorids. These are members of Theropoda (Fig. 1), a group that also includes *Tyrannosaurus* and *Allosaurus*. With a wildly projecting cassock perched atop a short, deep skull, long arms with three-fingered hands, each finger opposable and sharply taloned, and long, muscular, presumably fast-running hind legs, *Oviraptor* and its close relatives have the gestalt of a horrible nightmare. In this case, however, looks deceive. The front of the skull of these two-metre-long dinosaurs was formed into a toothless beak, the bones of the skull roof and the braincase contained a complex pattern of air sinuses, and paired peg-like projections occupied the midline of the palate (Fig. 2). What these unusual theropods ate is still not precisely known⁷.

As described by Norell *et al.*, the extraordinary preservation of the Ukhaa Tolgod *Oviraptor* owes a great deal to rapid death and burial in what must have been a powerful sandstorm, so sudden that we are left with the impression of an animal freeze-framed in the act of nest brooding. The specimen is largely complete, missing only the skull, parts of the hindlimb and the vertebral column. The arms are held back and somewhat laterally as if protecting the periphery of the nest. The belly, as indicated by the paired pubic bones, is positioned directly over the nest, while the hind legs are tightly folded, suggesting that brooding consisted in squatting above the eggs.

Beneath the *Oviraptor* skeleton are 15 eggs laid in a circular pattern; Norell *et al.* calculate that the nest may have originally contained as many as 22. Egg shape and eggshell architecture are fully consistent with eggs referred to *Oviraptor* known from other material, although there is no evidence of colonial nesting as suggested for several other kinds of dinosaur. It is not yet known whether any of these eggs contain embryonic material.

Astonishing as this new *Oviraptor* specimen is in its own right, its ultimate importance will emerge from the kinds of questions it solves and, more so, from those that it raises. For example, it extends the distribution of avian-style nest brooding down the evolutionary tree into the realm of non-avian dinosaurs for the first time. Furthermore, it opens up a wealth of research avenues on dinosaurian life histories, which are particularly poorly known among non-avian theropods. What is the relationship between egg size, clutch size and adult size at reproductive maturity in this and other dinosaurs? What is the behavioural and ecophysiological significance of positioning eggs in a circular pattern?

Combining information from this new specimen with that from the newly discovered embryonic and existing subadult and adult *Oviraptor* material, we can also investigate the morphometric changes with growth in this species and compare them with those in other theropods. Likewise, possible differences between the bones, joints and tissues of embryos, young and adults become open to study. The answers to these kinds of questions, when put into their evolutionary context⁸, should provide the basis for translating the reproductive biology, growth and development of individual dinosaurs into the stuff of evolutionary dynamics in this important group of Mesozoic tetrapods. □

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Blood and iron

ALL iron rusts in the end. Even the finest paints and platings merely postpone the evil day. Some simply cheat — a crafty marine paint turns rust white to make it less obvious. Now Daedalus is fighting back. Just as iron rusts spontaneously in a wet oxidizing environment, he says, so there should be some reducing environment in which rust reverts spontaneously to iron.

Such an antirusting reaction would probably be as mild, slow and relentless as rusting itself. Daedalus reckons it could be incorporated into paint. An antirusting paint would not merely act as a barrier to water and oxygen, or react with them pre-emptively to guard the metal beneath; it would actively reverse rusting as fast as it happened.

One obvious rust reducer is carbon monoxide. It is the active reagent of the blast furnace; at red heat it rapidly reduces iron ore (which is merely geological rust). Even at room temperature it has an intimate iron chemistry. This is why it is poisonous — it combines with the iron in haemoglobin, giving a bright red complex. DREADCO's chemists have invented a paint loaded with haemoglobin. Exposed to carbon monoxide, it absorbs the gas and flushes a lurid cherry red. The chemists are spraying it experimentally onto steel sheets, and leaving them out to rust. With luck, the carbon monoxide in the paint will reverse the rusting as fast as it occurs. When it is all used up, the paint will turn the purplish colour of venous blood. The sheets will then be put back into carbon monoxide, when the paint will recover its vivid colour and protective action.

Haemoglobin releases its carbon monoxide in bright light, disengaging the molecule in a somewhat energetic state. With a little chemical tweaking, the complex may be persuaded to trap the energy of daylight quite efficiently, and use it for very powerful rust reduction. The final product, DREADCO's 'Reverse Rust' paint, will be snapped up by engineers and householders alike. It can be painted straight onto rusty surfaces; it will last for ages; and when it finally gets exhausted it will change colour in the very places that need reactivation. You won't even have to scrape it off and repaint the surface: just smear or spray on a rejuvenator loaded with carbon monoxide in dissolved or complexed form. Objects up to about the size of a car (say) could be restored most easily by placing them in carbon monoxide gas. Those who commit suicide by running their car engine in a closed garage would journey to the next world in a bright and rustless vehicle.

David Jones

Explosive New Zealand mistletoe

SIR—Many flowers of the mistletoe *Peraxilla tetrapetala* (Loranthaceae) in New Zealand open from the bottom (*f* in the figure) rather than the top (*d*); Kuijt¹ called this an “unsolved mystery . . . we cannot even guess at the meaning of this bizarre performance.” Here we report that flower buds of *P. tetrapetala* and *P. colensoi* open from the top only when twisted by a bird, a form of ‘explosive’ flower opening common in Africa but previously unknown in Australasia. Kuijt’s “bizarre performance” is simply the consequence of flowers not being visited by birds. However, pollination in *Peraxilla* is possible without birds: unvisited flowers sometimes self-pollinate when the petals undergo abscission from the bottom.

The closely related *Alepis flavida* may show how explosive opening evolved. Its flowers open unaided, but birds twist buds to try and open them early. Another close relative, *Trilepidea adamsii*, is believed extinct, but historical paintings and herb-

arium sheets show an even more specialized explosive mechanism than in *Peraxilla*.

We discovered explosive flower opening in both endemic New Zealand *Peraxilla* species when we noted that flower buds bagged for hand pollination almost never opened (3/394 for *P. tetrapetala*, 1/82 for *P. colensoi*). The petals remained fused at the top, while eventually undergoing abscission from their base (*f* in the figure). Field observations of unbagged flowers revealed that they were opened by two native honeyeaters (Meliphagidae): tuis (*Prothemadera novaeseelandiae*; *c* in the figure) and bellbirds (*Anthornis melanura*).

Both pollinators twisted only ripe buds; neither bird visited flowers that had already opened. Nectar production schedules discourage repeat visits, as *Peraxilla* buds already contain 70–98% of all the nectar they will ever produce. Exclusion experiments showed that the dependence of *Peraxilla* species on birds is not total, as 22% of unopened flowers set seed.

In *A. flavida*, bagged flowers opened normally without the assistance of a bird. However, we saw a bellbird twist *A. flavida* buds in an unsuccessful attempt to hasten their opening; this suggests how explosive opening could have evolved. Birds benefit by being able to forage more efficiently, because buds have not had their nectar harvested by any other bird (or insect): tamper-proof twist-top fast food for birds. The mistletoe may benefit through more faithful pollinator attention.

These discoveries in *Peraxilla* led us to reconsider the extinct *Trilepidea adamsii*, once congeneric with *Peraxilla* and *Alepis*. Study of pollination in extinct species is difficult, but paintings and herbarium sheets show previously overlooked features diagnostic of very complex explosive flowers. In the African *Tapinanthus*³, ripe flower buds carry slits (fenestrae), which are probed by a bird’s beak, causing the flower to unzip down that side. The stigma swings outwards towards the bird’s head. The painting of *Trilepidea* by Osborne⁴ shows flowers exactly matching this

pattern, as do herbarium sheets (for example, 4 of 7 flowers on sheet AK103910). *Trilepidea* almost certainly had more complex explosive flowers than *Peraxilla*. Such specialization may have rendered *Trilepidea* more sensitive to reduced bird densities due to introduced mammalian predators, contributing to its rapid and puzzling⁵ decline.

Explosive flower opening is well known in other mistletoes⁶, including many of the 230 species in Africa³, and a few species in India⁷, Java, New Guinea and South America^{1,6}. However, this is the first report from Australasia. The New Zealand flora generally displays few specialized pollination mechanisms⁸. We have shown that the two endemic *Peraxilla* mistletoes, and probably also *Trilepidea*, show very specialized ornithophilous pollination. This has interesting biogeographical implications, as the New Zealand Loranthaceae are generally considered primitive⁹.

Our study also has implications for conservation. First, if outbreeding benefits *Peraxilla*, bird populations must be maintained, or cross-pollination becomes impossible. Current bird populations are insufficient in some areas to open more than a minority of *Peraxilla* flowers. Second, learning by the birds may be important. In the North Island, *Peraxilla* populations have been reduced markedly by Australian brushtail possums (*Trichosurus vulpecula*)¹⁰. In 1993–94, banding of trees to exclude possums allowed more extensive flowering, but the flowers were largely ignored by bellbirds (S. Dopson, personal communication), which may no longer know how to open them. Finally, posthumous recognition of explosive flowers in *Trilepidea* both sheds light on its extinction and increases our sadness at the fact.

Jenny J. Ladley

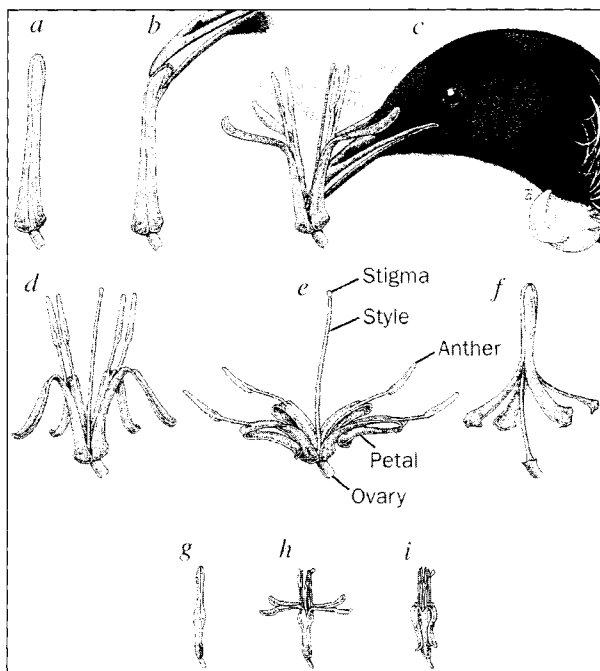
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Scientific Correspondence

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Explosive flower opening in the New Zealand mistletoe *Peraxilla colensoi* (a–f) and its possible precursor in *Alepis flavida* (g–i). *P. colensoi*: a, Unopened bud, mean length 47 mm. b, Bird (a tui) grasps the top of a ripe bud with its beak and twists. c, After 0.55 ± 0.10 s (mean $\pm$ 95% confidence interval, from 23 video recordings), the flower explodes open, flinging pollen from the already-dehiscid anthers, and the bird drinks nectar from the side or above, pollinating the flower. d, Four hours after opening, petals have folded back further. Birds ignore open flowers, which produce little nectar after the ripe-bud stage. e, Three days after opening, petals fold back through 180°. f, Without a twist from a bird, the top of the flower never opens, but 4–8 days after bud ripening the petals undergo abscission from the base and pull the anthers over the top of the stigma, aiding self-pollination. *Alepis flavida*: this species does not have explosive flowers, and flowers open unaided. However, impatient birds sometimes twist flowers to try and open them early. g, Unopened bud, mean length 20 mm. h, Open flower on day of opening. i, Petals fold back within 2 days of opening. Drawings by T. Galloway.

Asymmetric autocatalysis and amplification of enantiomeric excess of a chiral molecule

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THE homochirality of natural amino acids and sugars remains a puzzle for theories of the chemical origin of life^{1–18}. In 1953 Frank⁷ proposed a reaction scheme by which a combination of autocatalysis and inhibition in a system of replicating chiral molecules can allow small random fluctuations in an initially racemic mixture to tip the balance to yield almost exclusively one enantiomer. Here we show experimentally that autocatalysis in a chemical reaction can indeed enhance a small initial enantiomeric excess of a chiral molecule. When a 5-pyrimidyl alkanol with a small (2%) enantiomeric excess is treated with diisopropylzinc and pyrimidine-5-carboxaldehyde, it undergoes an autocatalytic reaction to generate more of the alkanol. Because the reaction involves a chiral catalyst generated from the initial alkanol, and because the catalytic step is enantioselective, the enantiomeric excess of the product is enhanced. This process provides a mechanism by which a small initial imbalance in chirality can become overwhelming.

In earlier studies of asymmetric autocatalytic reactions^{19–21}, the enantiomeric excess (e.e.) of chiral products has always been lower than that of the chiral catalysts. In our studies²² of the enantioselective addition of dialkylzincs to aldehydes using chiral β -aminoalcohols^{23,24} and piperazines²⁵, we have discovered that the pyrimidine-containing secondary alcohol, 2-methyl-1-(5-pyrimidyl)propan-1-ol (**1**), is an efficient asymmetric autocatalyst. We find that with only a small e.e., (*S*)-**1** catalyses the enantioselective addition of diisopropylzinc to pyrimidine-5-carboxaldehyde (**3**) (ref. 26) to yield (*S*)-**1** with significantly higher enantiomeric enrichment.

When a mixture with 5% e.e. of (*S*)-**1** (*S*-isomer: *R*-isomer = 52.5:47.5) was treated as an asymmetric autocatalyst (20 mol%) with aldehyde **3** and diisopropylzinc, pyrimidyl alcohol (*S*)-**1** was obtained with a 62% yield as a mixture of the newly formed product **1** and the catalyst (*S*)-**1** (run A1; Table 1). The amount of (*S*)-**1** in the catalyst increased by a factor of 4.1. It is surprising that the enantiomeric excess of the mixture of the newly formed product **1** and the catalyst **1** has increased to 39% (*S*-isomer: *R*-isomer = 69.5:30.5) (Fig. 1). This shows that (*S*)-**1** with 55% e.e. (note; >5% e.e.) was newly formed in 42% yield as a result of the asymmetric autocatalytic reaction of catalyst (*S*)-**1** (Table 1, run A1; Fig. 1). The reaction was performed successively, with the products of one round serving as the reactants for the next, resulting in an overwhelming imbalance between the two stereo isomers (Table 1).

In a similar manner, starting from the chiral catalyst (*S*)-**1** with even lower enantiomeric excess (2% e.e., *S*-isomer: *R*-isomer = 51:49) (20 mol%), asymmetric autocatalytic reaction of **1** and the amplification of the e.e. of **1** are also observed (series B in Table 1, runs 1–5). The e.e. of (*S*)-**1** has increased successively from 2% to 10%, 57%, 81% and 88% (runs 1–5). The amount of (*S*)-**1** of the initial catalyst (2% e.e., run B1) has increased by the factor of 942 times (run B5). When (*S*)-**1** with 88% e.e. was

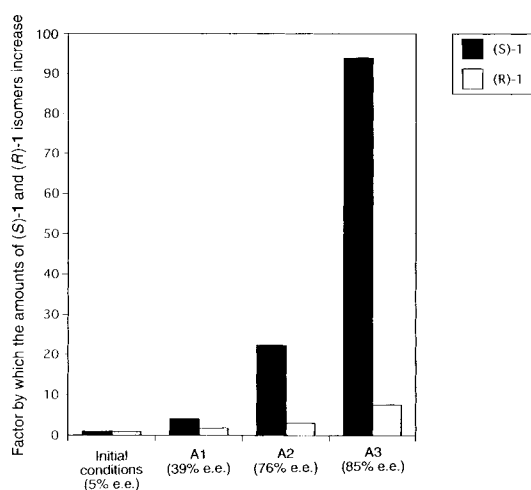


FIG 1. Asymmetric autocatalysis of chiral pyrimidyl alkanol (**1**). Runs A1–3 correspond to Table 1. The enantiomeric excess of (*S*)-**1** increases from 5 to 89% e.e. without the use of additional chiral auxiliaries. During the reactions (runs A1–3), the (*S*)-**1** increases by a factor of 94 times, while (*R*)-**1** increases by a factor of only eight times.

employed as asymmetric autocatalyst, the e.e. of the mixture of catalyst and the product was also 88% (run B5). Thus in series A and B, the low e.e. of (*S*)-**1** was autocatalytically amplified to 88–89%, and the amount of (*S*)-**1** was increased by a factor of 942 and 1,674.

On the other hand, when the opposite enantiomer, (*R*)-**1**, with 10% e.e. was employed as an asymmetric autocatalyst, the *R*-enantiomer of the mixture of the catalyst and the newly formed **1** was obtained in comparable enantiomeric excess (53% e.e.) (run C1; Table 1). When the asymmetric autocatalyst was (*R*)-**1** with 30% e.e., (*R*)-**1** increased to yield a mixture of the catalyst and the product with 72% e.e. (run C2). In addition, the enantioselectivity of a racemic **1** as autocatalyst was below the detection level (run D; Table 1). These results show that the configurations of the asymmetric autocatalyst, (*S*)- or (*R*)-**1**, determine the configuration of the product, (*S*)- or (*R*)-**1**. In other words, (*S*)- or (*R*)-**1** do work as asymmetric autocatalysts.

We propose the following reaction scheme for asymmetric autocatalysis of (*S*)-**1** (Fig. 2). Pyrimidyl alcohol (*S*)-**1** reacts with an equimolar amount of diisopropylzinc to form *in situ* the chiral isopropylzinc alkoxide **2** (characterized by proton NMR). Compound **2** catalyses the enantioselective addition of diisopropylzinc to aldehyde **3** to yield **2** with increased e.e. Subsequent

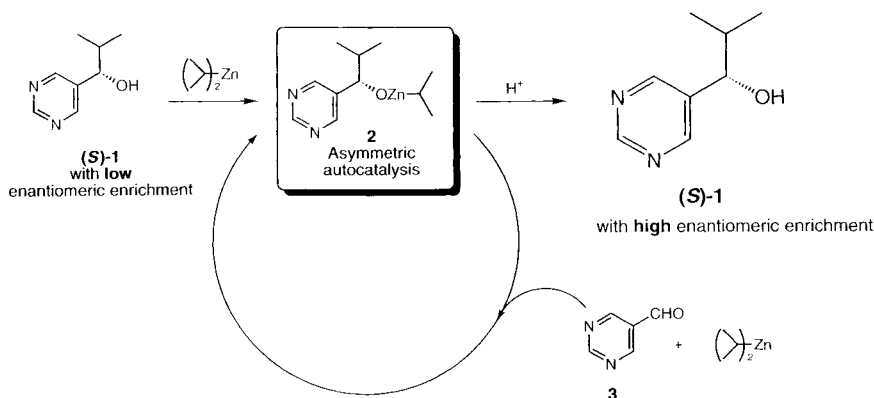


FIG. 2 Proposed reaction scheme of asymmetric autocatalysis of (*S*)-**1**.

TABLE 1 Asymmetric autocatalysis of chiral pyrimidyl alcohol **1**

Run*	Catalyst 1 (% e.e.)	Time (h)	Mixture of catalyst (1) and product 1		Factor by which the amount of (S)- 1 has increased	Newly formed product 1 [†]	
			Yield (%)	e.e. (%)		Yield (%)	e.e. (%)
Series A [‡]							
A1	5 (S)	74	62	39 (S)	4.1	42	55 (S)
A2	39 (S)	60	86	76 (S)	22	66	87 (S)
A3	76 (S)	68	80	85 (S)	94	60	88 (S)
A4	85 (S)	66	86	89 (S)	413	66	90 (S)
A5	89 (S)	60	81	89 (S)	1,674	61	90 (S)
Series B [‡]							
B1	2 (S)	74	46	10 (S)	2.5	26	16 (S)
B2	10 (S)	91	75	57 (S)	13	55	74 (S)
B3§	57 (S)	96	80	81 (S)	61	60	89 (S)
B4	81 (S)	70	75	88 (S)	239	55	90 (S)
B5	88 (S)	77	79	88 (S)	942	59	88 (S)
Series C							
C1	10 (R)	70	81	53 (R)		61	67 (R)
C2	30 (R)	65	79	72 (R)		59	86 (R)
Run D	Racemate	55	68	BDL		48	BDL

* Molar ratio. Pyrimidine-5-carboxaldehyde (**3**): diisopropylzinc: catalyst **1** = 1:1.2:0.2. All reactions were reproducible.

[†] The amount of pyrimidyl alcohol **1** after subtracting that of **1** used as a catalyst from that of total pyrimidyl alcohol.

[‡] In each series, the mixture of catalyst and the newly formed product are used as a catalyst of the next run.

§ As an example, details of the experimental procedure, and of the calculation of yield and e.e. are given for run B3. Pyrimidyl alcohol **1** (31.7 mg (0.208 mmol), 57% e.e., containing (S)-**1** (24.9 mg) and (R)-**1** (6.8 mg) in toluene (49 ml) and diisopropylzinc (1.2 ml of 1 M toluene solution, 1.2 mmol) was stirred for 20 min at 0 °C, and then a toluene solution (1.8 ml) of pyrimidine-5-carboxaldehyde (**3**) (112.7 mg, 1.04 mmol) (ref. 26, purified by sublimation) was added at 0 °C. This reaction mixture was stirred for 96 h at 0 °C, and was then quenched by the addition of 1.0 M hydrochloric acid (5 ml) and saturated aqueous sodium hydrogen carbonate (15 ml) at 0 °C. The mixture was filtered using celite and the filtrate was extracted with ethyl acetate. The extract was dried over anhydrous sodium sulphate and allowed to evaporate until dry. Purification of the crude product using thin-layer chromatography yielded pyrimidyl alcohol **1** (127.3 mg)—a mixture of the newly formed alcohol and the catalyst alcohol (31.7 mg). Analysis of the product by high-performance liquid chromatography using a chiral column (Daicel Chiralcel OD) showed it has 81% e.e. (see run B3); it therefore must consist of (S)-**1** (115.4 mg) and (R)-**1** (11.9 mg). The amount of newly formed alcohol **1** is 127.3 – 31.7 = 95.6 mg (0.628 mmol, 60% yield), which consists of (S)-**1** (115.4 – 24.9 = 90.5 mg) and (R)-**1** (11.9 – 6.8 = 5.1 mg). The newly formed alcohol thus has an enantiomeric purity of 89% e.e.

|| Below the detection level.

hydrolysis of **2** gives (S)-**1** with amplified e.e. (Fig. 3). Even when the catalyst has an e.e. of just 5%, the newly formed product (that is, **1**) had enantiomeric excess of 55%.

It seems conceivable that the reaction reported here may be an example of the scheme proposed by Frank⁷. The symmetry breaking by spontaneous crystallization reported by Kondepudi²⁷, which involves indirect inhibition, is the only other

possible experimental realization of this scheme so far, and that involved physical rather than chemical processes. But the detailed mechanism of the autocatalytic steps in our reaction, and in particular, whether an inhibitory mechanism is present, remains to be clarified. □

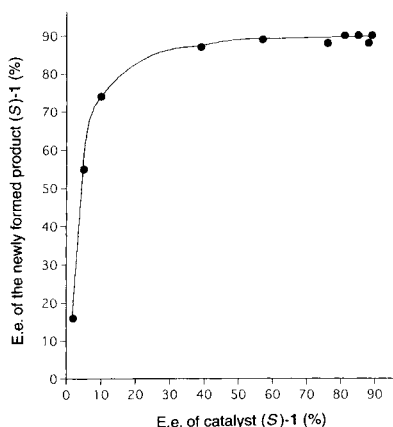


FIG. 3 Relation between the enantiomeric excess (e.e.) of catalyst (S)-**1** and that of the newly formed product (S)-**1** in asymmetric autocatalytic reaction of (S)-**1**.

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Continental climate response to orbital forcing from biogenic silica records in Lake Baikal

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CHANGES in insolation caused by periodic changes in the Earth's orbital parameters provide the primary forcing for global ice ages¹⁻⁶. But it is not clear to what extent the climates in continental interiors are controlled directly by regional variations in insolation and to what extent they are driven instead by the highly nonlinear response of the oceans and ice sheets. Here we investigate this question using the record of biogenic silica in Lake Baikal as a proxy for climate change in this high-latitude mid-continental region. We find a good correlation between this record and that of marine oxygen isotopes⁴. Over the past 250 kyr the Baikal record exhibits both a strongly nonlinear component (manifested in a 100-kyr periodicity) and weaker direct-insolation components (manifested in the 41-kyr (obliquity) and 23- and 19-kyr (precession) orbital cycles). These results show that even though extreme continental climates such as this are influenced directly by insolation variations, they are dominated by the nonlinear rhythm of the oceans and ice sheets.

Although studies of ocean and ice-sheet records have shown that they vary at the same frequencies as orbital parameters, comparison with climate changes in continental interiors has been hampered by the scarcity of long climate records on land. Records of continental climate parameters, such as dust⁷ and pollen⁸, are commonly found in marine cores, but isolating the

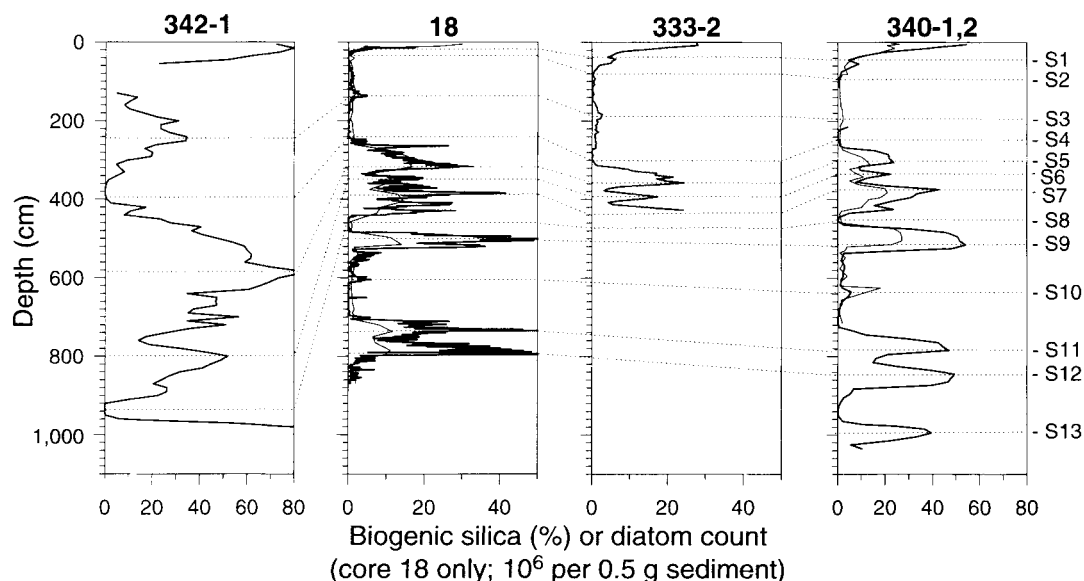
response of continental interiors in these records is difficult. Several well-known continental records correlate with the marine $\delta^{18}\text{O}$ record (SPECMAP⁹), including the magnetic susceptibility record in Chinese loess^{9,10} and the $\delta^{18}\text{O}$ of calcite in Devils Hole, Nevada¹¹. The former, however, is strongly influenced by the Asian monsoon, and the latter is influenced by Pacific moisture.

The relatively high-latitude (52–56° N), continental-interior location of Lake Baikal (Siberia) is an ideal setting in which to address questions of continental palaeoclimate¹². Perhaps no other place on Earth is as far removed from the influence of both oceans and former ice sheets. Baikal is thus an end-member location in which to compare the relative importance of direct insolation forcing with that of nonlinear ocean/ice-sheet effects. Our 250-kyr record from Baikal of biogenic silica (bSiO₂) probably reflects changes in diatom productivity in the lake.

Profiles of bSiO₂ content against depth (Fig. 1) contain features that are clearly correlative among core sites on Academic ridge in Baikal, despite differences in sedimentation rates. These correlations are entirely consistent with independent ones made from magnetic-susceptibility profiles¹³ and from lithological properties. Upper parts of cores from these sites, relatively well dated by accelerator mass spectrometry (AMS) radiocarbon¹⁴, are related to the last glacial-interglacial cycle. The uppermost, Holocene, parts of cores are diatomaceous mud in which bSiO₂ increases upwards, with some fluctuations (for example, S1 in Fig. 1). The late Pleistocene, glacial parts of the cores contains little or no bSiO₂. The transition between these two types of sediment consistently occurs¹⁵ at about 13 kyr ago. The bSiO₂ record of the last glacial-interglacial transition demonstrates the influence of climate on bSiO₂ and serves as an analogue for similar transitions deeper in the cores. Time-depth functions for this glacial half-cycle also provide reasonable average sedimentation rates ($\sim 5.1 \text{ cm kyr}^{-1}$ for Academic ridge) for undated lower parts of cores¹³.

Because of the highly dilute, oligotrophic waters of Baikal¹⁶, diatoms are the primary microfossils preserved in the sediments. Although total productivity of the lake is controlled by picoplankton (mostly cyanobacteria)¹⁷, diatoms dominate the larger phytoplankton group. Thus the amount of bSiO₂ preserved in sediments is a useful productivity index, which has been used in

FIG. 1 Biogenic silica content of sediment (weight per cent) plotted against depth at three sites on Academic ridge, and one site (342) nearby at the mouth of Maloe More, Lake Baikal. Unless otherwise noted, the analytical method was that of Carter and Colman¹⁵, based on that of Mortlock and Froelich³¹. The light line for sites 18 and 340-2 are from a secondary method²⁰, using an extraction solution 25% as strong, which gives correlative but consistently lower results (L. Z. Granina, personal communication). Heavy line for site 18 represents diatom frustules counted in smear slides (in millions per 0.5 g of sediment; M. A. Grachev and Ye. Likhoshway, personal communication). Records are composites of trigger and piston cores, the latter of which were commonly missing the uppermost section of sediment¹³. Note differences in bSiO₂ scales. Dotted lines (labelled at the right-hand side



of figure) show visual correlations between distinctive features of the profiles. Numbers after core sites refer to specific cores. Core site locations: 342: 53.3993° N, 107.5882° E; 18: 53.5594° N, 108.0131° E; 333: 53.6530° N, 108.1558° E; 340: 53.6672° N, 108.3610° E.

several previous studies^{18–20}. As in most oligotrophic lakes^{21,22}, only a small fraction of the total primary bSiO₂ in surface waters is preserved in the sediments. In marine sediments, selective dissolution of diatoms clearly affects preserved diatom assemblages, but bSiO₂ content is still a first-order indication of overall diatom productivity²³. Dissolution of diatoms is controlled by water chemistry, temperature and sedimentation rate. In the Holocene sections in Baikal cores, diatoms are relatively abundant and are mostly well preserved. Despite relatively low amounts of bSiO₂ in glacial-age sediments, it seems unlikely that dissolution is the primary control on the relative amounts of diatoms preserved, for the following reasons: (1) colder surface water during glacials would tend to inhibit dissolution compared to interglacials, opposite to observed relative abundances; (2) given the present dilute nature of the lake water, it is unlikely that glacial waters were significantly more under-saturated; (3) some interstadial peaks in biogenic silica occur in intervals that are dominated by small, fragile *Cyclotella* sp.; and (4) some cores, including those examined in detail here, have nearly constant sedimentation rates through glacial and interglacial times owing to a balance between terrigenous and biogenic sedimentation.

We therefore interpret the bSiO₂ record in Baikal as primarily a diatom productivity signal, with possible second-order dissolution effects. In addition to uncertainties about dissolution, however, the precise link between climate and diatom productivity is not known. Possible factors include surface-water temperature, length of the ice-free season, turbidity and nutrient (including Si) cycling. Whatever the exact links between climate, diatom productivity and dissolution, the variations in bSiO₂ since the Last Glacial Maximum clearly indicate dominant control by climate.

The bSiO₂ record from Baikal can be compared with two end-member models of long-term climate variation (Fig. 3): (1) the distinctly nonlinear (with respect to orbital forcing) marine record (SPECMAP) of global ice volume⁴, and (2) summer temperature predictions of an energy balance model driven by insolation changes²⁴. Although the latter takes into account land and water distribution, heat capacity and albedo, it does not include feedback mechanisms, ice sheets, or convective heat transport; its predictions can thus be thought of as potential temperatures in the absence of such nonlinear processes.

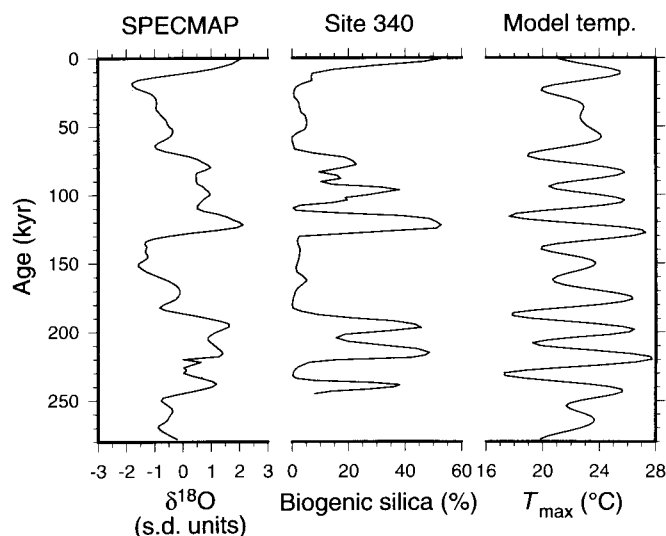


FIG. 2 Comparison of the bSiO₂ record from site 340 (this work) with the SPECMAP⁴ $\delta^{18}\text{O}$ record and with maximum summer temperatures (at 60° N, 100° E) derived from an energy-balance model driven by changes in solar insolation²⁴. Timescale for site 340 is from CORPAC²⁵ correlation to SPECMAP (see text); it corresponds to a nearly constant sedimentation rate of 4.0 cm kyr⁻¹, modified by internal adjustments of >5 kyr.

Site 340 was chosen for these comparisons because: (1) it is clearly the longest record (Fig. 1); (2) the sampling interval for bSiO₂ (10 cm) yields the same time resolution (~2 kyr by our initial age model for site 340) as that for the SPECMAP record, thus requiring neither smoothing nor interpolation; and (3) detailed magnetic properties for site 340 provide both assurance of completeness and comparisons with the results of Peck *et al.*¹³. To quantify the correlation between the bSiO₂ record at site 340 and these two end-member climate records, we used an inverse-correlation method (CORPAC²⁵) (Fig. 2). The results are not sensitive to our initial age estimates based on a sedimentation rate of 5.1 cm kyr⁻¹ at site 340. The optimal correlation coefficient with SPECMAP (0.84, 71% shared variance) is extremely high for two proxies reflecting two unrelated parameters in two entirely different systems. Despite obvious similarities between the bSiO₂ record and model temperatures, especially during interglacial times, the optimal correlation coefficient between the two is lower (0.55). Although some of the same frequencies and magnitudes are apparent in the latter two records, direct correlation is hampered by probable lags between model temperatures (forcing) and bSiO₂ (response). For this reason, correlation of model temperatures to the bSiO₂ record on the SPECMAP-correlation timescale is low (0.12). Additional support for our timescale comes from correlation of the relative magnetic intensity record for Baikal for the past 84 kyr with those of well dated marine cores²⁶.

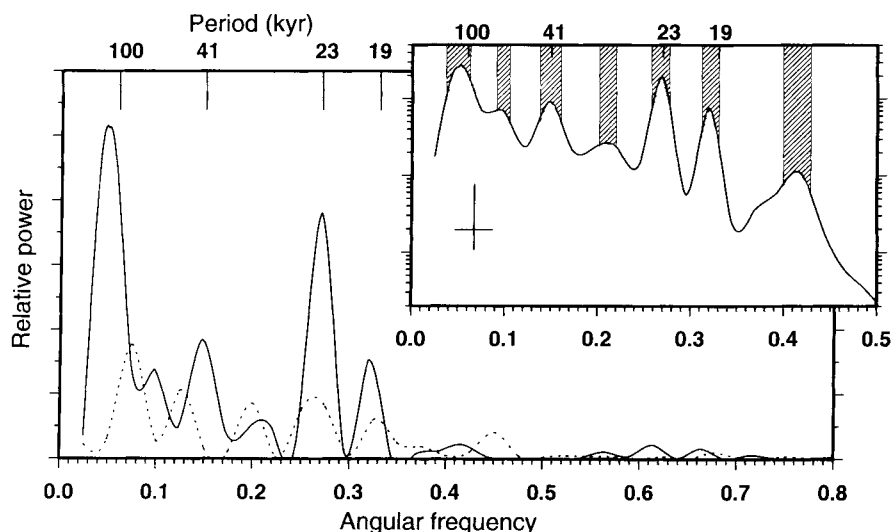
Spectral analysis of the bSiO₂ record (Fig. 3) shows that both the constant-sedimentation-rate model and the SPECMAP-correlation model contain frequencies close to those for orbital variations. The improved spectra over the initial constant-sedimentation-rate ages are the result of correlation to SPECMAP, and therefore do not validate the correlation. However, the combination of well dated changes in bSiO₂ during the last glacial-interglacial cycle and dominance of near-orbital periodicities in the spectra for the constant-sedimentation-rate model justify correlation to SPECMAP. Some uncertainty is introduced into the spectral analysis by the fact that the bSiO₂ record is only 2.5 times as long as the dominant cycle (~100 kyr) and by the fact that the constraint of zero bSiO₂ causes a slight 'clipping' of the record, which can affect the power contained in lower-frequency bands^{24,27,28}. But because of the size of the 100-kyr peak and the minor degree of clipping, neither of these effects is likely to alter our major conclusions.

The large amount of variance in frequencies near the 100-kyr eccentricity cycle is strikingly similar to the marine record, in which this band typically accounts for twice as much variance as the obliquity and precession bands combined⁶. The apparent dominance of the 100-kyr cycle suggests that the same nonlinear processes that amplify this cycle for the oceans and ice sheets^{5,6} also influence the climate of continental Asia. Despite its distance from the ocean and former ice sheets, Baikal is 'downwind' from the former Eurasian ice sheets, albeit by more than 1,700 km. Our results suggest that continental interiors are well integrated into the global climate system, so that no part of the Earth system is isolated from the nonlinear ocean/ice-sheet response to orbital forcing.

Despite the strength of the 100-kyr cycle, large amounts of variance appear near the precession cycles of 23 and 19 kyr and significant, but smaller, amounts of variance are associated with frequencies near the 41-kyr obliquity cycle (Fig. 3). Thus direct insolation forcing is clearly part of the Baikal bSiO₂ record despite the strong influence of the nonlinear 100-kyr cycle. Details of the bSiO₂ record also suggest direct insolation forcing as an important part of the Baikal climate history. The large range in bSiO₂ variation within interglacial times (isotope stages 5 and 7) is notably similar to the variation in summer insolation during those times^{29,30} and to the model temperatures²⁴ derived from those insolation variations (Fig. 2).

The variance in the obliquity band is smaller than that in the precessional band. This contrasts with SPECMAP⁴ and Chinese

FIG. 3 Spectral analysis (using CORPAC²⁵) of the bSiO₂ record from site 340, using the initial linear (5.1 cm kyr⁻¹) timescale (dotted line) and the SPECMAP-correlation timescale (solid line). Multi-taper methods³² produced essentially identical results. Power units are arbitrary. Inset, spectral data for the SPECMAP-correlation timescale on a logarithmic scale; the horizontal part of the cross represents the bandpass filter and the vertical part represents error bars. Portions of the curve peaks that exceed the 95% confidence interval are indicated by shading.



loess^{9,10} records and is somewhat unexpected, because summer insolation at high northern latitudes (typically 65° N; refs 2–5) is thought to be critical in initiating ice-sheet growth. Also, because the effect of obliquity on insolation increases with latitude, obliquity dominates total high-latitude insolation. Precession dominates low-latitude insolation, as well as high-latitude seasonal extremes^{8,24,30}. The relative weakness of the 41-kyr obliquity peak in the Baikal record suggests that the influence of climate processes driven by low-latitude insolation may be felt at higher latitudes. Also, because northern maritime land masses are thought to be necessary lead factors in the initiation of glaciation during obliquity cycles^{6,24}, our data suggest that northern interiors such as Baikal may be less important than maritime ones in this initiation.

It appears that the palaeoclimate response in relatively high-latitude, continental Asia, as reflected in the bSiO₂ record of Baikal, is dominated by a 100-kyr cycle, in a manner similar to the nonlinear response to orbital forcing exhibited by global ice volume and various ocean parameters. Despite the sensitivity of Baikal's setting to direct insolation forcing and evidence for the influence of precession and obliquity cycles, the many feedbacks and nonlinearities of the global climate system appear to strongly influence the record of such continental interiors, amplifying the 100-kyr cycle there as well as in the oceans and ice sheets. Our results suggest an integrated, unified response of the global climate system to orbital forcing. □

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Hafnium–tungsten chronometry and the timing of terrestrial core formation

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THE accretion of the Earth and Moon within the solar nebula is thought^{1–3} to have taken 50 to 100 million years. But the timing of formation of the Earth's core has been controversial, with some^{4,5} proposing that it took place within the first 15 Myr of Earth's accretion history and others^{6,7} proposing that it occurred after 50 Myr of accretion. Meteorite chronometry based on the ¹⁸²Hf–¹⁸²W system has the potential to resolve this debate, as segregation of a metal core from silicates should induce strong fractionation of hafnium from tungsten. Here we report tungsten isotope compositions for two iron meteorites, two carbonaceous chondrites, and a lunar mare basalt. We see clear ¹⁸²W deficits in both iron meteorites, in agreement with previous results^{4,5}. But the data for chondrites are inconsistent with the hypothesis of early core formation, suggesting that both this event and the formation of the Moon must have occurred at least 62 ± 10 Myr after the iron meteorites formed.

Hafnium and tungsten are both highly refractory elements that are assumed to be present in chondritic relative proportions in the Earth. Hafnium is a lithophile element whereas tungsten

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is a moderately siderophile element that should strongly partition into metal phases during metal/silicate segregation, resulting in a large fractionation of Hf/W between stony and iron meteorites or between the silicate mantle and metallic core of a terrestrial planet. Consequently, if core formation took place when there was still ^{182}Hf (half-life, 9 Myr) on Earth, the W remaining in the silicate mantle would develop an excess abundance of ^{182}W relative to that of chondrites^{4,5}. Conversely, if core formation were late, after all ^{182}Hf had decayed, the W isotope composition of the silicate Earth would be identical to that found in chondrites.

The relationship between W and Hf isotope compositions in the present-day bulk Solar System or in chondrites (CHOND) can be expressed as follows:

$$\left(\frac{^{182}\text{W}}{^{184}\text{W}}\right)_{\text{CHOND}} = \left(\frac{^{182}\text{W}}{^{184}\text{W}}\right)_{\text{BSSI}} + \left[\left(\frac{^{182}\text{Hf}}{^{180}\text{Hf}}\right)_{\text{BSSI}} \times \left(\frac{^{180}\text{Hf}}{^{184}\text{W}}\right)_{\text{CHOND}}\right] \quad (1)$$

where BSSI is the initial value for the bulk Solar system and $^{180}\text{Hf}/^{184}\text{W}$ (atomic ratio) = $1.18 \times \text{Hf/W}$ (weight ratio). If the small contribution of s-process ^{182}Hf (ref. 8) is disregarded, $(^{182}\text{Hf}/^{180}\text{Hf})_{\text{BSSI}}$ is primarily a function of the amount of freshly synthesized r-process Hf introduced to the solar nebula before its collapse, and the time that elapsed between production and collapse. Equation (1) defines a linear relationship between $^{182}\text{W}/^{184}\text{W}$ and $^{180}\text{Hf}/^{184}\text{W}$ that may be obtained by measurements on undisturbed minerals of early Solar System materials such as chondrites. The $(^{182}\text{Hf}/^{180}\text{Hf})_{\text{BSSI}}$ would then correspond to the slope of such an internal isochron. However, useful limits on this parameter can be obtained from bulk meteoritic material, as in this study.

In the simplest model, the $^{182}\text{Hf}/^{180}\text{Hf}$ at the time of core formation (CF) of a planet such as the Earth that may have started with chondritic Hf/W and at some point segregated and maintained two systems, core and bulk silicate Earth (BSE), is given by the differences in W isotope composition and Hf/W ratio between the BSE and chondrites:

$$\left(\frac{^{182}\text{Hf}}{^{180}\text{Hf}}\right)_{\text{CF}} = \left\{ \left(\frac{^{182}\text{W}}{^{184}\text{W}}\right)_{\text{BSE}} - \left(\frac{^{182}\text{W}}{^{184}\text{W}}\right)_{\text{CHOND}} \right\} \left\{ \left(\frac{^{180}\text{Hf}}{^{184}\text{W}}\right)_{\text{BSE}} - \left(\frac{^{180}\text{Hf}}{^{184}\text{W}}\right)_{\text{CHOND}} \right\}^{-1} \quad (2)$$

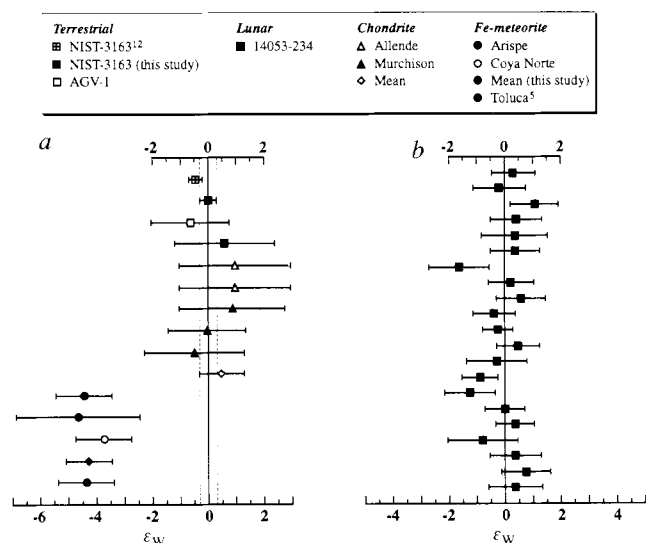


FIG. 1 a, ϵ_{W} values of terrestrial and lunar samples, chondrites and iron meteorites for this study (Table 1), calculated relative to the mean of 21 independent measurements of NIST-3163 W standard, shown in b. The average value of Toluca⁵ is also included, converted to $^{182}\text{W}/^{184}\text{W}$ assuming $^{183}\text{W}/^{184}\text{W} = 0.467126$ (ref. 12) and, subsequently, to ϵ_{W} relative to the mean of NIST-3163 of this study.

Obviously the W isotope composition of the core ($^{182}\text{W}/^{184}\text{W}$)_{CF} cannot be measured directly, but it can be predicted from mass balance if the entire Earth has a chondritic Hf/W ratio. The W isotope composition of the core ($^{182}\text{W}/^{184}\text{W}$)_{CF} should then balance the W isotope composition of the BSE and produce an average weighted total-Earth W isotope composition that equals $(^{182}\text{W}/^{184}\text{W})_{\text{CHOND}}$.

The Hf isotope composition at the time of core formation $(^{182}\text{Hf}/^{180}\text{Hf})_{\text{CF}}$ can also be expressed as a function of $(^{182}\text{Hf}/^{180}\text{Hf})_{\text{BSSI}}$, the decay constant λ , and the amount of time that had elapsed since collapse of the solar nebula ΔT , as follows:

$$\left(\frac{^{182}\text{Hf}}{^{180}\text{Hf}}\right)_{\text{CF}} = \left(\frac{^{182}\text{Hf}}{^{180}\text{Hf}}\right)_{\text{BSSI}} \times e^{-\lambda \Delta T} \quad (3)$$

This yields the time between collapse of the solar nebula and core formation as:

$$\Delta T = -\lambda^{-1} \times \ln \left\{ \left(\frac{^{182}\text{Hf}}{^{180}\text{Hf}}\right)_{\text{BSSI}} \times \left\{ \left(\frac{^{182}\text{W}}{^{184}\text{W}}\right)_{\text{BSE}} - \left(\frac{^{182}\text{W}}{^{184}\text{W}}\right)_{\text{CHOND}} \right\} \left\{ \left(\frac{^{180}\text{Hf}}{^{184}\text{W}}\right)_{\text{BSE}} - \left(\frac{^{180}\text{Hf}}{^{184}\text{W}}\right)_{\text{CHOND}} \right\}^{-1} \right\} \quad (4)$$

Because of its very high first ionization potential, negative-ion thermal ionization mass spectrometry (NTIMS) has been the principal analytical technique for W (ref. 9). A recent study using NTIMS reported the first evidence of a deficiency in ^{182}W relative to terrestrial W in the iron meteorite Toluca⁴. An alternative method for determining the isotope composition of elements with high first ionization potential is multiple-collector inductively-coupled plasma mass spectrometry (MC-ICPMS)¹⁰⁻¹³. All the W isotope measurements reported here were performed with this new technique.

The W isotope compositions determined in five independent measurements (full chemistry included) of two carbonaceous chondrites, Allende (CV) and Murchison (CM), agree within analytical uncertainty (Table 1, Fig. 1). This W isotope composition is identical to that of the bulk silicate Earth (BSE) as represented by the W solution standard NIST-3163 and the USGS international rock standard AGV-1. Similarly, the $^{182}\text{W}/^{184}\text{W}$ ratio of an Apollo 14 low-Ti mare basalt, 14053-234, agrees within uncertainty with that of both the BSE and the carbonaceous chondrites (Table 1). In contrast, the two iron meteorites yield low $^{182}\text{W}/^{184}\text{W}$, corresponding to -4.5 ± 1.0 and $-3.7 \pm 1.0 \epsilon_{\text{W}}$ units for Arispe (IA) and Coya Norte (IIA) respectively, expressed as deviations in parts per 10^4 relative to the NIST-3163 W standard (Table 1, Fig. 1). These results confirm, and are in excellent agreement with, the NTIMS data for the Toluca meteorite⁵ ($\epsilon_{\text{W}} = -4.3 \pm 1.0$), suggesting that the observed ^{182}W deficit relative to BSE W is a common feature of iron meteorites. As the modification of W isotopes by neutron capture during space exposure is negligible¹⁴, the observed ^{182}W deficit is most likely the consequence of segregation of metal to form the iron meteorite protolith within the lifespan of ^{182}Hf .

Jacobsen and Harper⁵ argued for a short (< 15 -Myr) interval of core formation based on the W isotope composition of the iron meteorite Toluca. However, this conclusion was based on the assumption of a low $(^{182}\text{Hf}/^{180}\text{Hf})_{\text{BSSI}}$ of $\sim 10^{-5}$ (ref. 15), such that the W isotope composition of chondritic material would not change greatly with time. Thus the data for Toluca would provide a close estimate of the W isotope composition of chondrites. Clearly, the W isotope compositions of carbonaceous chondrites and iron meteorites are very different (Fig. 1), and the $(^{182}\text{Hf}/^{180}\text{Hf})_{\text{BSSI}}$ must be high. Its minimum value can be calculated from equation (1) using the measured $^{182}\text{W}/^{184}\text{W}$ of iron meteorites, as this must represent an upper limit for the bulk Solar System initial $(^{182}\text{W}/^{184}\text{W})_{\text{BSSI}}$. The Hf/W ratio of CM type chondrites is 1.33 (ref. 16), corresponding to a $(^{180}\text{Hf}/^{184}\text{W})_{\text{CHOND}}$ of 1.57. Assuming this is precise to at least

TABLE 1 Tungsten isotope data

Sample	$^{182}\text{W}/^{184}\text{W}$	ϵ_{W}
Terrestrial		
NIST-3163*	0.86494 ± 2	-0.46 ± 0.2
NIST-3163†	0.86498 ± 3	0.00 ± 0.3
AGV-1	0.86492 ± 12	-0.64 ± 1.4
Lunar		
14053-234	0.86503 ± 16	0.58 ± 1.8
Chondrite		
Allende-1	0.86506 ± 18	$+0.98 \pm 2.0$
Allende-2	0.86506 ± 18	$+0.98 \pm 2.0$
Murchison-1	0.86505 ± 17	$+0.86 \pm 1.9$
Murchison-2	0.86498 ± 12	-0.05 ± 1.4
Murchison-2	0.86494 ± 16	-0.50 ± 1.8
Mean	0.86502 ± 7	$+0.47 \pm 0.8$
Iron meteorite		
Arispe-1	0.86459 ± 9	-4.45 ± 1.0
Arispe-2	0.86457 ± 19	-4.67 ± 2.2
Coya Norte	0.86466 ± 9	-3.73 ± 1.0
Mean	0.86461 ± 7	-4.27 ± 0.8

All samples processed using chemical techniques to be described elsewhere. Chondrite powders (1–5 g) were made from fresh 25 g pieces provided by the Smithsonian Institution. The iron meteorites were sawed as clean centre cubes from slabs in the University of Michigan meteorite collection, and leached with 6 ml of hot aqua regia three times before total dissolution. All the measurements were performed in static mode and utilized 1 μg to 150 ng of W. All the ratios were normalized to $^{186}\text{W}/^{184}\text{W} = 0.927633$ (ref. 12), and corrected for Os interferences at masses 184 and 186 by monitoring mass 188. The only currently unidentifiable spectral interference is a small baseline effect found in separated natural materials at mass 183. Total process blanks were $\leq 3\%$ of the measured W. An exponential law was used to correct for mass discrimination¹². All the quoted uncertainties are 2σ standard errors, and refer to the least significant digits in the case of $^{182}\text{W}/^{184}\text{W}$. The ϵ_{W} values were calculated relative to the NIST-3163† standard. ϵ_{W} is given by

$$\left\{ \left[\left(^{182}\text{W}/^{184}\text{W} \right)_{\text{meas}} - \left(^{182}\text{W}/^{184}\text{W} \right)_{\text{std}} \right] / \left(^{182}\text{W}/^{184}\text{W} \right)_{\text{std}} \right\} \times 10^4.$$

* From Lee and Halliday¹².

† Mean of 21 measurements conducted during this study.

$\pm 5\%$, from equation (1), the $(^{182}\text{Hf}/^{180}\text{Hf})_{\text{BSE}}$ is therefore $\geq (2.61 \pm 0.13) \times 10^{-4}$.

The Hf/W ratio of BSE is ~ 22 (ref. 17), entirely in keeping with W being moderately siderophile and estimates of $D^{\text{met/sil}}$ during core formation (where D is the distribution coefficient, or ratio of the concentrations, for metal and silicate liquids). The effect of a high Hf/W ratio, like that of the BSE, on W isotope compositions is shown in Fig. 2. If core formation on the Earth and the formation of the iron meteorites were contemporaneous, a residual Hf/W ratio of 22 would generate an easily resolvable $\epsilon_{\text{W}} \approx +90$ relative to carbonaceous chondrites. The later the formation of the core, the more closely the $^{182}\text{W}/^{184}\text{W}$ of the BSE would approach that of carbonaceous chondrites if the Earth accreted with chondritic Hf/W (Fig. 2). The earliest that core formation could have occurred, given all analytical errors and uncertainties in $(^{180}\text{Hf}/^{184}\text{W})_{\text{BSE}}$, is 62 ± 10 Myr after the formation of iron meteorites, or else the W isotope composition of the BSE would not be chondritic (Fig. 2). The data presented here are consistent with the model of Galer and Goldstein⁶, who suggested an accretion and core formation interval of 80 ± 40 Myr for the Earth using U–Th–Pb systematics. A similar result has been obtained by Allègre *et al.* (ref. 18). However, the Pb data are interpreted very differently by others⁵.

The W data present a highly robust constraint that holds true regardless of whether the W inventory of the BSE is affected by a late veneer. Heterogeneous accretion^{19–21} is generally considered to be a multi-stage process with the BSE inventory of W entirely dominated ($>99.9\%$) by late chondritic additions²². But even if this is the case, the effects of early core formation on W would still be resolvable in the BSE, exactly as in Fig. 2.

Qualitatively speaking this is because the greater the proportion of BSE W that comes from late additions, the greater the Hf/W of the mantle must have been before these additions, therefore, the greater the isotopic effect from this stage would be and the harder it would be to overprint the W isotope composition. If the entire Earth is chondritic with respect to Hf/W and is separated into two phases, BSE and core, with higher and lower Hf/W ratios respectively, the W isotope compositions and Hf/W ratios of core, BSE and total Earth (chondrites) must all define an isochron slope that is a function of the $^{182}\text{Hf}/^{180}\text{Hf}$ at the time of core formation, which in turn is a function of the age of the core (equation (2)). Additions of further chondritic material do nothing to alter the slope, that is, the W isotope composition and Hf/W ratio of BSE would be diluted proportionally. The predicted present-day W isotope composition of BSE would be resolvable higher than chondritic until ~ 60 Myr after the formation of iron meteorites, regardless of the fraction of W in BSE that is late, given a BSE Hf/W of 22.

The data presented here also place important constraints on the origin of the Moon. The Hf/W ratio of the lunar mantle is probably similar to that of the BSE given the similarity in W/U ratio²³. Therefore, radiogenic W would be expected for lunar mantle if the Moon formed within ~ 60 Myr of iron meteorites. Not surprisingly, the W isotope composition of the silicate Moon is also chondritic and identical to the Earth (Fig. 1). The Moon may have formed from mixed debris created by a giant impactor that hit the Earth with a glancing blow, after terrestrial core formation^{23–26}. Ringwood²⁶ even proposed that formation of the Moon post-dated the addition of the chondritic veneer to the Earth's mantle on the basis of the presence of enriched magmas preserved as rare glasses, that contrast with mare basalts. Therefore, even without the W isotope data, the age of the Moon has to post-date the iron meteorites by at least 62 ± 10 Myr. This is consistent with the oldest reliable ages for lunar rocks^{3,27}, but is more difficult to reconcile with the recent Sm–Nd age of 4.56 ± 0.07 Gyr (ref. 28), obtained from a clast of a lunar breccia. If this age and uncertainty are correct, the Moon has to have formed 4.50 ± 0.01 Gyr ago.

The W data provide clear support for the long-held assumption that the Earth must have chondritic relative proportions of refractory elements, or else the W isotope composition in BSE would differ from chondritic, regardless of when core formation

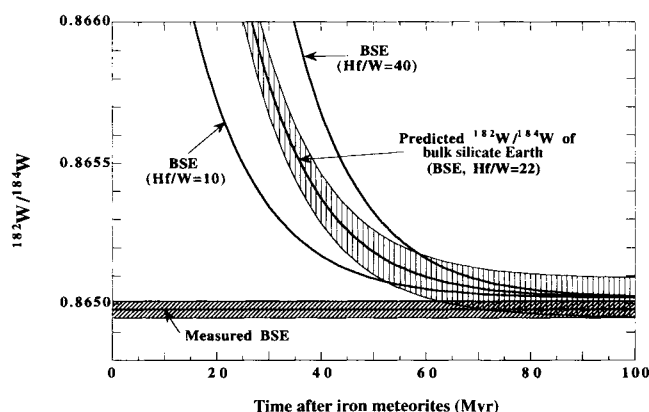


FIG. 2 Predicted $^{182}\text{W}/^{184}\text{W}$ of bulk silicate Earth (BSE) versus the putative time (in Myr after the formation of iron meteorites) of core formation, given that the BSE Hf/W is 22 (ref. 17). The uncertainties are shown by the width of the shaded band. Two separate calculations assuming Hf/W ratios of BSE of 10 and 40 are also shown, representing extremes for the W depletion factors suggested for BSE (ref. 17). The measured BSE $^{182}\text{W}/^{184}\text{W}$ is shown for comparison. The minimum time of core formation, when the uncertainties of the predicted and the measured BSE $^{182}\text{W}/^{184}\text{W}$ start to overlap, is 62 Myr after the formation of meteorites, with an uncertainty of ~ 10 Myr, given that the BSE Hf/W ratio is between 10 and 40.

occurred. Recent studies²⁹ of the ⁵³Mn-⁵³Cr system (half-life, 3.7 Myr) indicate that the Cr isotope composition of the Earth and Moon are similar but distinct from (less radiogenic than) chondritic compositions. Manganese is more volatile than Cr (ref. 22), so the unradiogenic Cr isotope composition of the Earth can be explained if the Earth accreted from volatile depleted material with low Mn/Cr. However, core formation, being late, had no effect on Cr or W isotope compositions. □

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A nesting dinosaur

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A SPECTACULAR fossil specimen that suggests the presence of an avian type of nesting behaviour in oviraptorids, a clade of non-avian maniraptoran theropods, is reported here. The substantial evidence indicating that birds are a type of theropod dinosaur has led to copious discussion concerning the origin and possible presence of advanced avian reproductive behaviour in non-avian dinosaurs. Although the inference of behaviour from fossils is problematic, some remarkable discoveries, such as the incontrovertible evidence of dinosaur nests¹, and more controversial claims made on the basis of dinosaur nesting grounds² and juvenile morphology³, hint at the occurrence of advanced reproductive behaviour in a variety of non-avian dinosaurs. But there is no direct fossil evidence implying advanced parental systems such as those found in modern birds. The closest associations between presumed parents and nests occur in oviraptorid dinosaurs from Late Cretaceous deposits of the Gobi Desert^{4,5}. The specimen described here is the first preserved well enough to determine its precise relationship with the nest. It is a large oviraptorid positioned over a nest of oviraptorid eggs in the same posture taken by many living birds when brooding. This provides the strongest evidence yet for the presence of avian brooding behaviour in non-avian dinosaurs.

Other *Oviraptor* discoveries have been found associated with nests^{4,5}, including the first discovery of *Oviraptor* at the Flaming Cliffs in 1923⁴, and it has been suggested previously that perhaps these individuals were defending or incubating their nests⁵. At the time of the original discovery in 1923, the eggs were thought to belong to *Protoceratops andrewsi*, the most common dinosaur in those deposits. This led to the eponymous suggestion that *Oviraptor* died while scavenging the eggs. The recent discovery of an oviraptorid embryo⁶ within the type of egg associated with

the *Oviraptor philoceratops* holotype suggests instead that this individual's proximity to the nest was related to parental care rather than to predation.

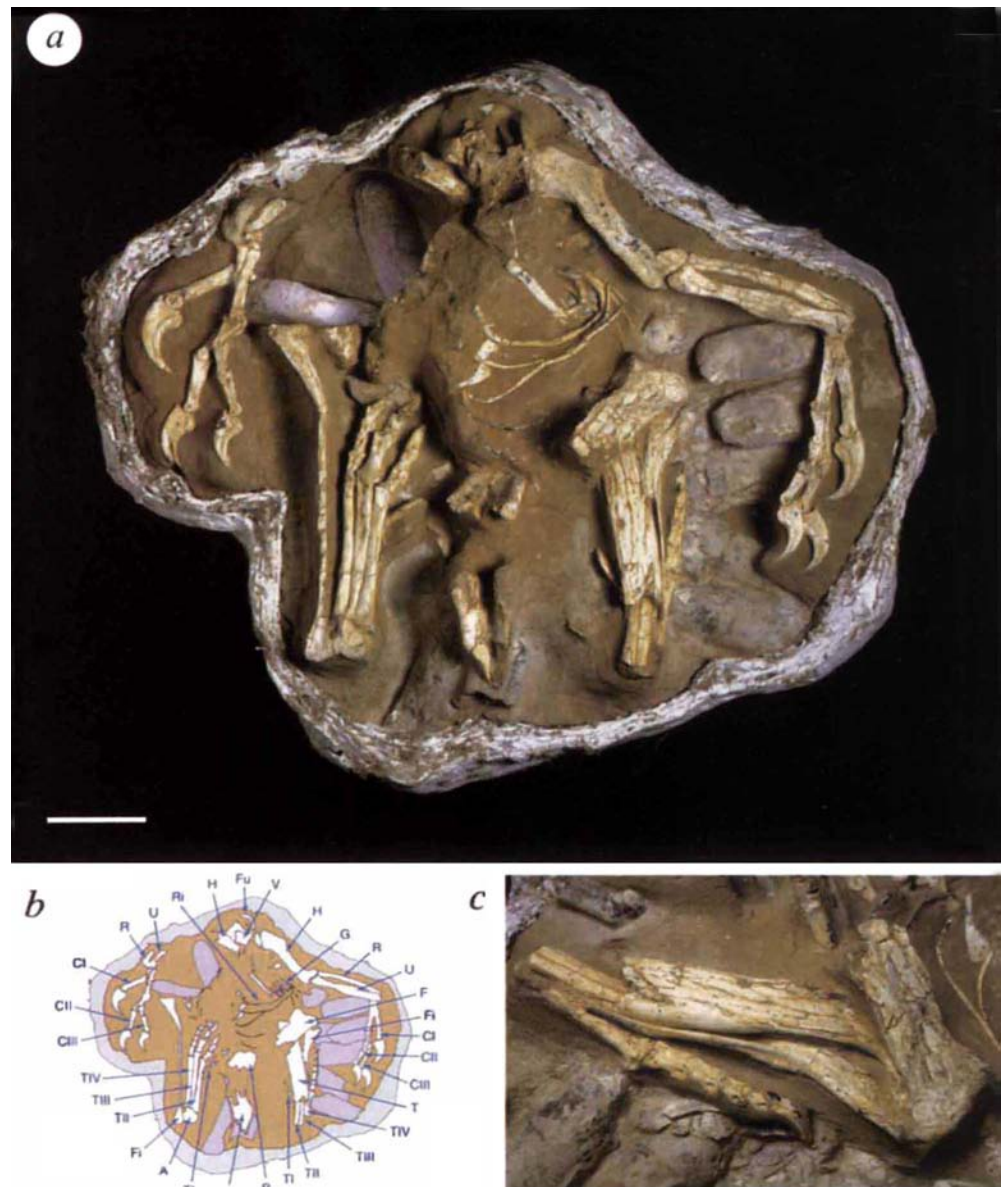
The specimen (IGM 100/979) (Fig. 1) was collected at Ukhaa Tolgod, a Late Cretaceous fossil locality in South Central Mongolia^{7,8}, during the 1993 segment of the Mongolian Academy of Sciences/American Museum of Natural History of Paleontological Project. To preserve spatial relationships definitively the entire specimen was collected in a single large block. No eggs were exposed on the surface, indicating that the entire nest as preserved was collected.

At Ukhaa Tolgod, remains of oviraptorids are the most common theropod elements encountered, rivalling ankylosaurs as the most common dinosaur discovered at this locality⁷. Like most specimens from Ukhaa Tolgod, the specimen shows no evidence of transportation after death, and is preserved in a facies hypothesized to be deposited by large sandstorms⁷. The specimen is of a large individual, although it is not outside the range of Ukhaa Tolgod oviraptorids. The skull, vertebrae, tail and dorsal pelvic bones, and proximal parts of both hindlimbs are missing, yet the majority of the remaining elements including the gastralia and ribs are preserved (Fig. 1).

The maniraptoran affinity of this specimen is shown by the presence of a semilunate carpal that is firmly secured to metacarpals I and II⁹. The clavicles are fused forming a stout furcula, a feature typical of oviraptorids¹⁰. IGM 100/979 has a forward-pointing pubis and metatarsal III is not pinched proximally by II and IV; digit three in the hand is gracile as is typical of many maniraptorans. Differences in manual proportions have been used to differentiate oviraptorid taxa^{10,11}. In *Oviraptor* and *Conchoraptor*, digits II and III are subequal in length and longer than digit I, whereas all three digits are nearly equal in length in *Ingenia*. Furthermore, the digits of *Oviraptor* are longer and thinner than in other oviraptorids and the taxon uniquely displays large, laterally compressed, recurved claws, with extremely large flexor tubercles as expressed in IGM 100/979. The specimen displays several pathologies, including a right ulna that was broken and healed during life.

IGM 100/979 is the best preserved and most complete oviraptorid specimen of any yet found on a nest, and offers the first evidence of the precise position of the skeleton to the nest (Fig. 2). Both hindlimbs are tightly folded (Fig. 1c), with the feet and the lower legs nearly parallel to one another. The feet lay atop and adjacent to eggs on the inner perimeter of the circle defined

FIG. 1 a, Specimen photograph of IGM 100/972. Scale bar, 10 cm. b, Schematic map of the specimen. Exposed eggs are indicated in blue. Cl-III, Manual digits; F, femur; Fi, fibula; Fu, furcula; G, gastralia; H, humerus; I, ischium; P, pubis; R, radius; Ri, ribs; S, scapula; T, tibia; TI-IV, tarsal elements; U, ulna; V, vertebrae. c, Detail of the right pes of IGM 100/972 showing close association between the *Oviraptor* and the eggs.



by the nest (Fig. 1c). The pubis lies in the direct centre of the nest, and the ischia (which are fused distally) lay more posteriorly atop the posteriormost eggs. Anteriorly, gastralia just posterior to the shoulder girdle lie in contact with eggs. The front limbs are directed posteriorly, with both arms wrapped around the nest. The claws on the hands are directed inward. Evidence of soft tissue preservation, unusual for sandstone matrices, is apparent at the tips of the manual claws (Fig. 3).

The eggs in the nest are arranged in a circular pattern, with the broad end of the egg pointing towards the centre of the nest. In places they occur in two levels. Fifteen eggs are visible; however, additional eggs are undoubtedly present in areas that could not be prepared without damage to the skeleton. From the spacing and distribution of the visible eggs, it can be estimated that about 22 eggs filled the nest, a number typical of nests that can be referred to oviraptorids¹². Individual eggs measure 18 cm long by 6.5 cm wide although post-mortem vertical compression may influence these measurements to a small degree. Egg surfaces are ornamented with small ridges that run parallel to the long axes, and their shape can be characterized as elongatoolithid¹³. Eggshell morphology is identical to an egg containing an oviraptorid embryo⁶ and those associated with other oviraptorid skeletons^{4,5}.

Although other nests containing eggs with an identical eggshell morphology are common in the area, none was discovered close enough to suggest the occurrence of a communal nesting colony as has been postulated for the ornithomimid *Maiasaura*^{2,14}.

Other factors could possibly account for the association found in IGM 100/979. The nature of fossil preservation at Ukhaa Tolgod (no apparent post-mortem transportation), the near life pose of the oviraptorid skeleton on the nest, and the positive association of these eggs suggest strongly that this is not a chance occurrence. Another possibility is that the animal perished while in the act of laying eggs in the nest. This seems obviated by the lack of eggs inside the body cavity. Furthermore, the neat systematic arrangement of this and other oviraptorid nests



FIG. 2 Reconstruction of *Oviraptor* on nest shortly before death.

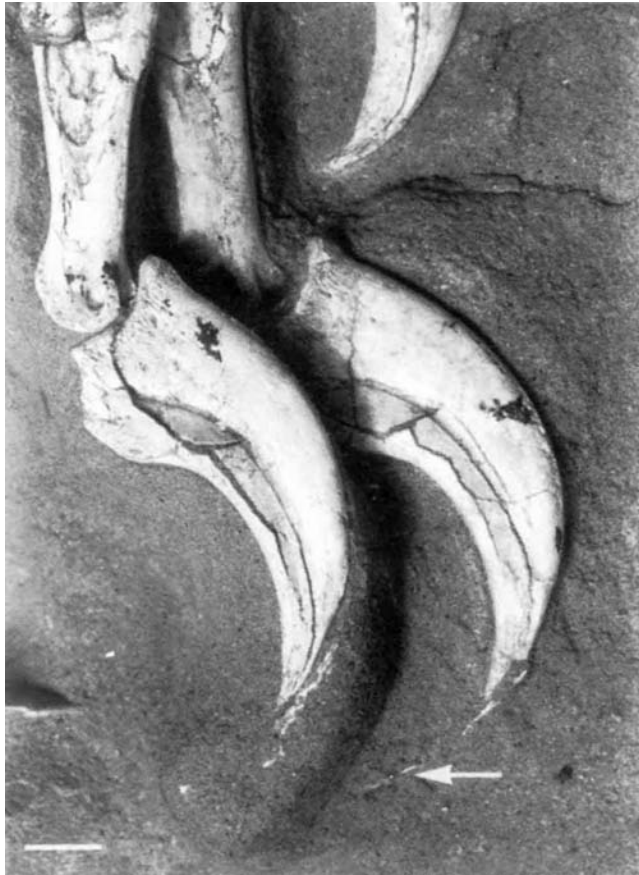


FIG. 3 Detail of manual claw of digit I. Arrow shows traces of soft tissue preservation and extent of horny covering of the ungual. Scale bar, 1 cm.

implies that the eggs were manipulated¹⁵ by the parents into a specific configuration after laying as in living birds.

Brooding behaviour (by which we refer only to the behaviour of sitting on nests) is exhibited by living birds to varying degrees and in diverse ways among the different groups¹⁵. However, in all but exceptional cases (nest parasites, mound builders) an individual (usually both sexes in neognaths¹⁶) rests directly upon the eggs for prolonged periods of time during their maturation. In palaeognaths the nest is typically attended by males¹⁶ (in both sexes in ostriches¹⁵). This behaviour is associated with thermoregulatory incubation. Very exceptionally, some extant ectotherms are also known to brood their nests (pythons¹⁷) where this behaviour is also correlated with maintenance of a constant, higher than ambient temperature during incubation. Although strongly suggestive, this does not imply that brooding behaviour and endothermy are necessarily correlated. Brooding behaviour may have developed as a mechanism for shading the eggs (common in arid-land birds^{15,18}), or as an advanced system of egg protection for ground nests that was later co-opted within birds in parallel with their metabolic evolution. Their phylogenetic distribution indicates that both endothermy and brooding behaviour are primitive characteristics among modern birds.

The occurrence of multiple specimens of large oviraptorids associated with nests suggests that (1) they were the parents of the nests, (2) that like other archosaurs they habitually stayed close to the nest, and (3) with the addition of IGM 100/992, that they brooded their nests. Unfortunately the nature of the fossil record limits the testing of these hypotheses. However, the sedimentary context of IGM 100/979 and its death position relative to the nest provide compelling evidence of an *Oviraptor* sitting on a clutch of eggs in a position homologous to the

brooding posture displayed by many modern birds. This finding provides the strongest evidence yet that modern avian brooding behaviour evolved long before the origin of modern birds and among non-avian maniraptoran theropods. □

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Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons *in vitro*

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DOWN'S syndrome (DS) or trisomy 21 is the most common genetic cause of mental retardation¹. Development of the DS brain is associated with decreased neuronal number and abnormal neuronal differentiation^{2–7}, and adults with DS develop Alzheimer's disease^{8,9}. The cause of the neurodegenerative process in DS is unknown. Here we report that cortical neurons from fetal DS and age-matched normal brain differentiate normally in culture, but DS neurons subsequently degenerate and undergo apoptosis whereas normal neurons remain viable. Degeneration of DS neurons is prevented by treatment with free-radical scavengers or catalase. Furthermore, DS neurons exhibit a three- to fourfold increase in intracellular reactive oxygen species and elevated levels of lipid peroxidation that precede neuronal death. These results suggest that DS neurons have a defect in the metabolism of reactive oxygen species that causes neuronal apoptosis. This defect may contribute to mental retardation early in life and predispose to Alzheimer's disease in adults.

Primary mixed cultures of human neurons and astrocytes were established from 16–19 weeks' gestation fetal DS and normal cerebral cortex as previously described¹⁰. DS and normal human neurons survived plating and elaborated axons and dendrites to a similar extent during the first 5–7 days in culture (Fig. 1a). However, after 7 days in culture, DS cultures began to show neurodegenerative changes, including neuronal shrinkage, vacu-

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olization and neurite fragmentation, that were not observed in normal cultures. From 7 to 14 days in culture, extensive loss of neurons and neuritic processes occurred in DS but not in normal control cultures (Fig. 1a). After 14 days in culture, neuronal viability in DS cultures was markedly reduced to $41 \pm 3\%$ of the value at day 7 (mean $\pm$ s.e.m. of seven independent experiments, $P < 0.01$ by ANOVA) (Fig. 2), whereas neuronal viability in normal cultures did not decline ($111 \pm 7\%$). In contrast, astrocyte viability was unimpaired in DS cultures, as determined by immunocytochemical staining for glial fibrillary acidic protein (GFAP) (Fig. 1b) and quantification of astrocyte cell number

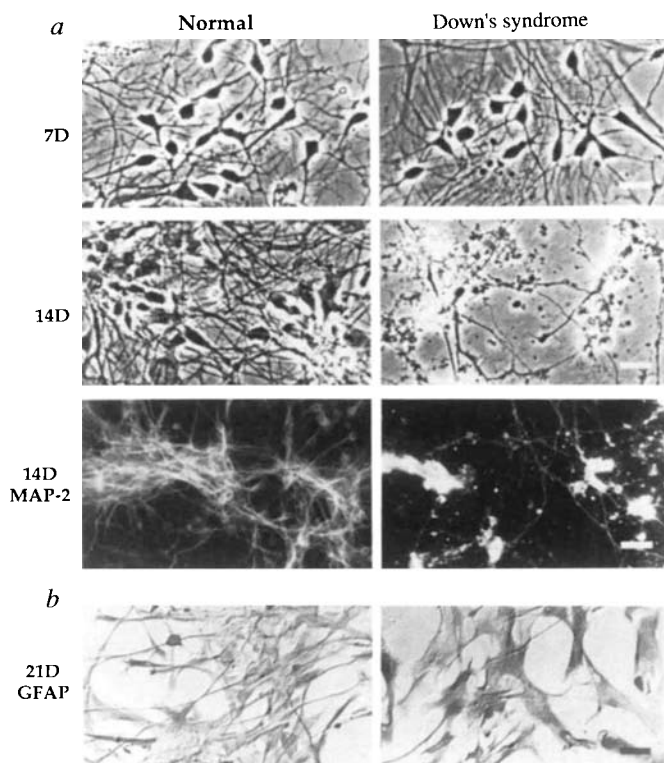


FIG. 1 Degeneration of Down's syndrome cortical neurons. a, Fetal cortical neurons from normal and Down's syndrome (DS) brains after 7 and 14 days in culture. DS and normal cortical neurons survive and develop well differentiated axonal and dendritic processes by 7 days in culture (7D). This is followed by profound degeneration of DS neurons after 14 days in culture (14D) with loss of MAP-2-immunoreactive processes (14D MAP-2). b, DS and normal astrocytes are similarly viable after 21 days in culture. Shown is immunocytochemical staining for GFAP. Scale bars: 7D and 14D, 20 μ m; 14D MAP-2 and 21D GFAP, 40 μ m.

METHODS. Primary human cortical cultures were established from the cerebral cortex of 16–19 weeks' gestation DS and normal fetal abortuses. Tissue is procured after signed consent in accordance with institutional guidelines. Cortical tissue samples were dissected free of meninges and dissociated by incubation with trypsin as previously described¹⁰. Cortical cells were plated on polylysine-coated glass coverslips in 24-well tissue culture plates at 200,000 cells per well and grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% supplemented calf serum (HyClone), 2 mM glutamine, 1 mM sodium pyruvate, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin. Cultures were fixed in 4% paraformaldehyde, 0.12 M sucrose in PBS, pH 7.0 for 30 min and permeabilized with 0.2% Triton X-100. For immunocytochemistry, fixed cells were incubated with primary antibody for 12 h at 4 °C (monoclonal antibodies: to MAP-2, clone AP20, 1:500, Sigma; to GFAP, clone GA5, 1:300, Boehringer Mannheim). The cells were then incubated with biotinylated anti-mouse IgG (1:200) for 1 h at room temperature, followed by ExtrAvidin-peroxidase (1:200) and diaminobenzidine. For immunofluorescence, rhodamine or fluorescein-conjugated anti-mouse IgG secondary antibodies were used (1:100). Degeneration of DS neurons was observed both in serum-containing medium and in serum-free medium containing N2 supplements.

(day 14:day 7, DS $313 \pm 48\%$; normal $298 \pm 36\%$). Thus, DS cortical neurons selectively undergo a degenerative process in culture.

Degenerating DS neurons exhibited membrane blebbing, chromatin condensation and DNA fragmentation characteristic of apoptosis¹¹ (Fig. 3a–c). Swollen neurons suggestive of necrosis appeared much less frequently in DS cultures. The number of apoptotic cells in DS cultures increased progressively from 7 to 14 days and accounted for most of the neuronal loss (Fig. 3d). In contrast, a low level of apoptosis occurred in normal cultures which did not increase from 7 to 14 days. Apoptosis of DS neurons was prevented by the protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D (Fig. 3e). These results suggest that DS neurons degenerate predominantly by a mechanism of programmed cell death.

Oxidative stress has been considered to be a possible contributory factor in the pathology of DS^{1,12,13}. To determine the role of reactive oxygen species (ROS) in DS neuronal degeneration, DS and normal cortical cultures were treated with antioxidants from days 7 to 14 in culture. The death of DS neurons was inhibited by the free-radical spin trap *N*-tert-butyl-2-sulphophenyl nitron (S-PBN), the free-radical scavengers vitamin E, butylated hydroxyanisole (BHA) and *N*-propyl gallate, and the glutathione precursor/radical scavenger *N*-acetyl-L-cysteine (NAC) (Fig. 2). In addition, catalase, an enzyme that hydrolyses hydrogen peroxide, inhibited the degeneration of DS neurons, whereas superoxide dismutase and albumin had no significant effect (Fig. 2). The nitric oxide synthase inhibitor nitroarginine (0.1–1 mM) and the broad spectrum excitatory amino-acid

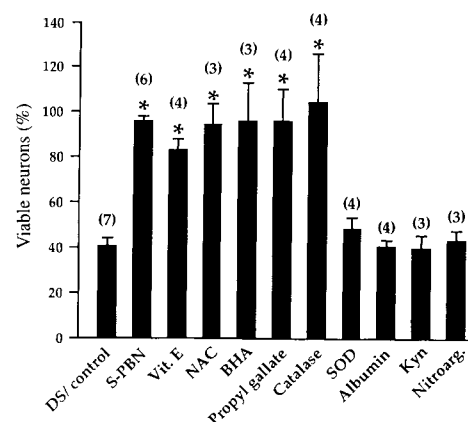


FIG. 2 Antioxidants inhibit the degeneration of DS neurons. DS cortical cultures were incubated in the presence or absence (control) of the indicated agents from days 7 to 14 in culture. Neuronal viability at day 14 is expressed as the per cent of the starting neuronal number at day 7 (100%). Untreated DS cultures (DS/control) show $41 \pm 3\%$ neuronal viability. Values are the mean $\pm$ s.e.m. of the number of independent experiments indicated in parentheses. * $P < 0.01$ relative to control by ANOVA with post-hoc Student–Newman–Keuls test.

METHODS. Drugs were added to DS cortical cultures at day 7, with fresh drug added every two days until day 14, when the cultures were fixed and immunocytochemically stained for neuron-specific tau (anti-tau, 1:1000, DAKO). Viable neurons with intact somas and neuritic processes (Fig. 1a) were scored by a blinded observer in 10 microscopic fields (0.8 mm² per field) per well in triplicate wells for each experiment (>450 neurons scored in controls). Drug concentrations: catalase 0.5 mg ml⁻¹ (10,000 U ml⁻¹); Cu/Zn-superoxide dismutase (SOD) 0.5 mg ml⁻¹ (2,000 U ml⁻¹); albumin 0.5 mg ml⁻¹; *N*-tert-butyl-2-sulphophenyl nitron (S-PBN) 100 μ M; butylated hydroxyanisole (BHA) 20 μ M; *N*-propyl gallate 5 μ M; *N*-acetyl-L-cysteine (NAC) 1 mM; vitamin E (Vit. E) 250 μ M; Nitroarginine (Nitroarg.) 100 μ M; Kynurenate (Kyn) 2 mM. BHA and Vit. E were dissolved in ethanol and diluted 1:200 into culture medium; this concentration of ethanol had no significant effect on neuronal viability when added alone. All other agents were made as 100 $\times$ stock solutions in sterile distilled water. SOD and polyethylene glycol-conjugated SOD had the same effect.

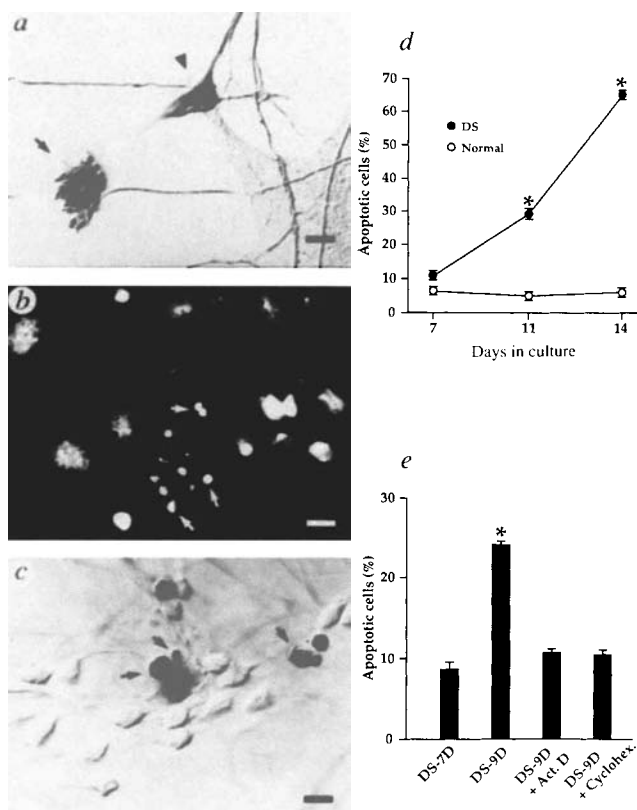
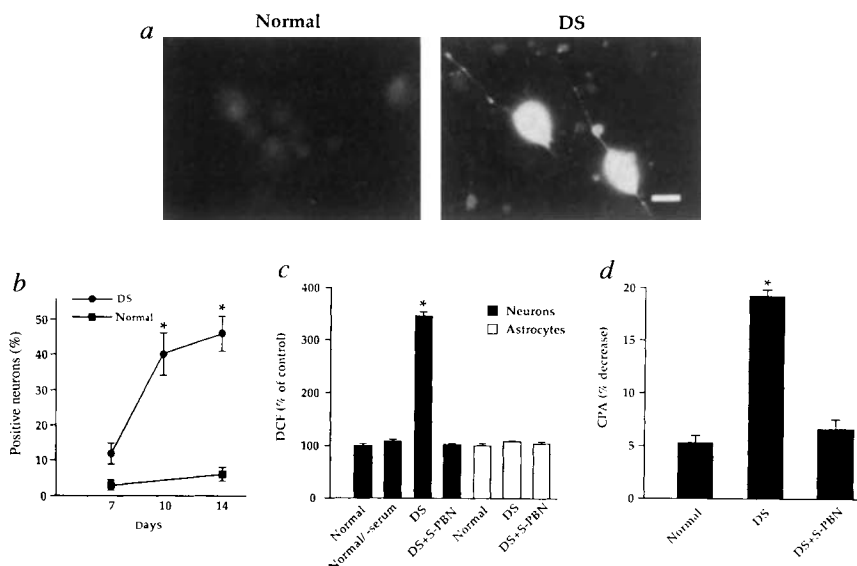


FIG. 3 Programmed cell death of DS neurons. **a**, Nomarsky microscopy of a 10-day DS culture showing a normal neuron (arrowhead) adjacent to a neuron exhibiting membrane blebbing characteristic of apoptosis (arrow). **b**, Chromatin condensation and nuclear fragmentation in degenerating DS neurons (arrows). Fluorescence micrograph of a 10-day DS culture stained with Hoechst dye No. 33258. **c**, DNA fragmentation in DS neurons (arrows). Shown are DS neurons stained by the TdT-mediated dUTP nick end-labelling (TUNEL) method and imaged with Nomarsky optics. The neuronal identity of TUNEL-positive cells was confirmed by immunocytochemical labelling for neuron-specific tau (not shown). **d**, DS but not normal cultures show a progressive increase in the number of apoptotic cells from days 7 to 14 in culture. **e**, Apoptosis in DS cultures is inhibited by actinomycin D (2 nM) and cycloheximide (4 μ M). Drugs were added at day 7 and apoptosis was determined at day 7 (DS-7D) and day 9 (DS-9D). Apoptotic cells are expressed as per cent of total neuronal number and represent the mean $\pm$ s.e.m., $n=4$. * $P<0.001$ by analysis of variance (ANOVA) with post-hoc Bonferroni test. Scale bars: **a**, **b**, 10 μ m; **c**, 20 μ m.

METHODS. Fixed cultures were stained by the TUNEL method using the Apoptag kit (Oncor). Chromatin staining of the nucleus was performed by incubation with 0.1 mg ml⁻¹ Hoechst dye No. 33258 in PBS for 10 min. Per cent of apoptotic cells was determined by counting apoptotic and non-apoptotic nuclei stained with Hoechst dye in both the culture medium and substratum of each well. Astrocytes were not counted. A similar time course of apoptosis was obtained by counting TUNEL-positive adherent cells.

FIG. 4 Generation of ROS and lipid peroxidation in DS neurons. **a**, Normal and DS cortical neurons were incubated at day 14 with 2,7-dichlorofluorescein diacetate, a fluorescent probe of intracellular ROS. Fluorescence microscopy shows that DS neurons but not normal neurons are intensely labelled. **b**, Progressive increase in ROS in DS neurons from days 7 to 14 in culture. The number of dichlorofluorescein (DCF)-positive neurons is expressed as per cent of the total neuronal number and represents the mean $\pm$ s.e.m., $n=15$. **c**, Quantification of ROS levels shows a significant increase in DS neurons which is inhibited by pretreatment with S-PBN. DCF fluorescence was measured by fluorescence-activated cell sorting (FACS) in 14-day DS and normal neuronal and astrocyte cultures, and after treatment of DS cultures from days 7–14 with 100 μ M S-PBN. Normal/–serum indicates normal cortical cultures induced to undergo apoptosis by serum withdrawal for 60 h. Values are expressed as per cent of the DCF level in normal cultures (100%) and represent the mean $\pm$ s.e.m., $n=5,000$ cells. **d**, Lipid peroxidation is increased in DS cortical culture and is inhibited by pretreatment with S-PBN. Lipid peroxidation is indicated by the per cent decrease in *cis*-parinaric acid (CPA) fluorescence in day 14 cultures measured by FACS. Values are the mean $\pm$ s.e.m., $n=10,000$. S-PBN did not significantly affect DCF or CPA fluorescence in normal cortical cultures. Scale bar in **a**, 10 μ m. * $P<0.001$ by ANOVA with post-hoc Bonferroni test.

METHODS. To determine ROS levels, cell suspensions (5×10^5 cells per ml) were washed with DMEM and incubated with 5 μ M dichlorofluorescein diacetate (Molecular Probes) for 1 h. Cells were washed again and 5 μ g ml⁻¹ propidium iodide was added. DCF fluorescence was determined in a Becton Dickinson Vantage FACS at excitation and emission wavelengths of 488 and 525 nm respectively. Gating out of



propidium-positive cells was performed to exclude debris and dead cells before data collection. To determine lipid peroxidation, cortical cell suspensions were incubated with 10 μ M CPA (Molecular Probes) for 1 h as described¹⁷. The cells were then washed, resuspended in DMEM, and 5 μ g ml⁻¹ propidium iodide was added. CPA fluorescence was determined in viable cells by FACS (excitation and emission wavelengths of 334–364 nm and 424 nm, respectively) immediately after incubation with CPA and 2 h later; the per cent decrease indicates the rate of lipid peroxidation. Astrocyte cultures were prepared as previously described²⁶.

receptor antagonist kynurenate (2 mM) did not significantly affect DS neuronal viability (Fig. 2). None of the treatments significantly affected neuronal viability in normal cortical cultures. These results suggest that ROS derived from hydrogen peroxide mediate the degeneration of DS cortical neurons.

To determine whether DS neurons generate increased levels of ROS, we used the dye 2,7-dichlorofluorescein diacetate which is permeable to cells and interacts with intracellular ROS to generate fluorescent 2,7-dichlorofluorescein (DCF)¹⁴⁻¹⁶. Fluorescence microscopy demonstrated markedly increased ROS levels in DS neurons but not in normal neurons (Fig. 4a). ROS levels in DS cultures increased progressively from days 7 to 14 (Fig. 4b) reaching a mean value of $346 \pm 7\%$ of the level of normal cultures, as determined by fluorescence-activated cell sorting ($n = 5,000$, $P < 0.001$ by ANOVA) (Fig. 4c). ROS levels were elevated in DS neurons that were morphologically intact and excluded propidium iodide (Fig. 4a, c), indicating that increased ROS generation precedes neuronal death. DS astrocytes did not show increased ROS levels (Fig. 4c), and elimination of microglia by treatment with leucine methyl ester did not prevent the degeneration of DS neurons (data not shown). Thus, DS neurons are the primary cellular source of increased ROS.

To confirm that increased free-radical generation causes cellular damage in DS cultures, lipid peroxidation was measured using a sensitive fluorimetric assay that is based on membrane incorporation of the fluorescent polyunsaturated fatty acid *cis*-parinaric acid (CPA)¹⁷. Peroxidation of CPA results in loss of fluorescence, providing a quantitative measure of lipid peroxidation. Lipid peroxidation in DS cortical cells was significantly greater than that detected in normal cortical cells (Fig. 4d). The free radical spin trap S-PBN inhibited the generation of ROS and prevented lipid peroxidation in DS cortical cultures (Fig. 4c, d), and protected against neuronal apoptosis (Fig. 2). These results suggest that a defect in the metabolism of ROS in DS neurons leads to apoptosis.

To determine whether the oxidative defect in DS neurons is common to other causes of neuronal apoptosis^{18,19}, we examined the effects of antioxidants on apoptosis induced by serum withdrawal²⁰ or staurosporine²¹. S-PBN (100 μ M), NAC (1 mM) and catalase (10,000 U ml⁻¹) did not protect against apoptosis of normal cortical neurons induced by serum withdrawal for 60 h or by treatment with 1 μ M staurosporine for 24 h (data not shown). Furthermore, serum withdrawal and staurosporine did not induce the increase in intraneuronal ROS levels observed in DS neurons (Fig. 4c). These results are in agreement with recent reports which suggest that elevated ROS is not the cause of all forms of apoptosis^{21,22}, and provide evidence that apoptosis of DS neurons is induced by a specific oxidative defect.

These experiments demonstrate that fetal DS neurons generate increased levels of ROS leading to neuronal apoptosis. Increased generation of ROS may contribute to abnormal brain development and mental retardation in DS. Neuronal depletion and structural abnormalities become evident during later gestational stages of DS brain development and early in postnatal life^{4,6,7,23}, consistent with our finding that DS neurons initially survive and differentiate in culture and then subsequently degenerate. These observations also raise the possibility that a neuronal oxidative defect may predispose to the early onset of Alzheimer's disease in DS individuals^{8,9}, consistent with increased oxidative damage in the brains of patients with Alzheimer's disease^{24,25}. If these findings in cell culture are confirmed *in vivo*, then the neuroprotective effects of antioxidants may provide an important therapeutic approach to mental retardation and the prevention of Alzheimer's disease in DS individuals. □

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Unaltered susceptibility to BSE in transgenic mice expressing human prion protein

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PRION diseases are transmissible neurodegenerative conditions of humans and animals. Prions consist principally of a post-translationally modified form of prion protein (PrP), PrP^{Sc}, which is partly protease resistant¹. Transmission of prion diseases between species is limited by a 'species barrier'² determined in part by the degree of sequence homology between host PrP and inoculated PrP^{Sc} (ref. 3) and by prion strain type⁴. The epidemic of bovine spongiform encephalopathy (BSE) in the United Kingdom and other countries has led to concerns that transmission to humans may occur by dietary exposure. BSE appears to be caused by a single strain, distinct from those of natural or experimental scrapie⁴, which is also seen in the new prion diseases of cats and ruminants that have presumably arisen from dietary BSE exposure⁴. Here we show that transgenic mice expressing human PrP in addition to mouse PrP can generate human PrP^{Sc} and 'human' prions. These mice therefore provide a model to study experimentally the species barrier limiting BSE transmission to humans. Incubation periods to BSE in transgenic mice are not shortened by expression of human PrP, and only mouse PrP^{Sc} is produced in response to such challenge.

We first studied mice that express both human and mouse PrP to determine whether, on challenge with Creutzfeldt-Jakob disease (CJD) inocula, they could produce human PrP^{Sc} and human prions. Two transgenic lines were used, Tg110 and Tg152 (ref. 5), with expression levels of human PrP of 50% and 200% of that seen in normal human brain, respectively⁶. Here we describe primary transmission and second passage of a single isolate of human prions from a CJD case (PDG170). We have also trans-

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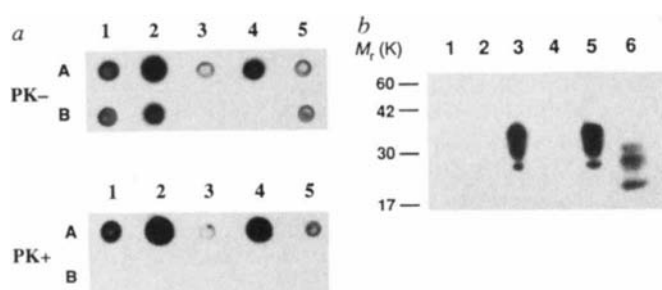


FIG. 1 Determination of the species type of PrP^{Sc} produced in transgenic mice expressing both human and mouse PrP, following inoculation with CJD. **a**, Immunodotblot of brain homogenates before and after treatment with proteinase K using monoclonal antibody 3F4, which detects human but not mouse PrP^{Sc} . A1 and A2, Tg152 mice of genotype $\text{HuPrP}^{+/+} \text{Prn-p}^{+/+}$; A3 and A4, Tg152 mice of genotype $\text{HuPrP}^{+/+} \text{Prn-p}^{0/0}$ following inoculation with CJD case PDG 170; A5, CJD case PDG 170; B1 and B2, age-matched Tg152 mice of genotype $\text{HuPrP}^{+/+} \text{Prn-p}^{+/+}$ inoculated with saline; B3, $\text{HuPrP}^{0/0} \text{Prn-p}^{+/+}$ (wild type); B4, $\text{HuPrP}^{0/0} \text{Prn-p}^{0/0}$ (PrP null); B5, normal human brain. PK +/– indicates with or without treatment with proteinase K (PK). **b**, Western blot of brain homogenates using monoclonal antibody 3F4, which detects human but not mouse PrP^{Sc} . Lanes 1 and 2, $\text{HuPrP}^{0/0} \text{Prn-p}^{+/+}$ (wild-type) mouse, before and after treatment with PK, respectively; 3 and 4, Tg152 mouse of genotype $\text{HuPrP}^{+/+} \text{Prn-p}^{+/+}$ inoculated with saline, before and after PK, respectively; 5 and 6, $\text{HuPrP}^{+/+} \text{Prn-p}^{+/+}$ inoculated with CJD case PDG 170, before and after PK, respectively. Numbered lines indicate positions of molecular size markers (kilodaltons).

METHODS. **a**, The method was modified from previous studies²⁶. Brain homogenates (10%) were prepared in a Dounce homogenizer in cold lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P40, 0.5% sodium deoxycholate in 10 mM Tris HCl, pH 7.4) and spun at 500g for 5 min at 4 °C. Supernatant (2 μl) was mixed with 2 μl SDS sample buffer (100 mM, pH 6.8, 2% SDS, 5 mM EDTA, 16% sucrose, Bromophenol blue) and dotted onto nitrocellulose (0.45 μm ; Biorad) filters in duplicate and left to air dry. Filters were washed in TBST (150 mM NaCl in 10 mM Tris HCl, pH 7.8, 0.05% Tween 20) for 10 min, and one of each pair of filters was treated with proteinase K at 50 $\mu\text{g ml}^{-1}$ for 2 h. Filters were then treated with 3 mM phenyl methylsulphonylfluoride for 20 min, followed by a 10-min incubation with 3 M guanadine thiocyanate. Filters were rinsed in TBST for 3 min and then blocked for 1 h using 5% 'blotto' (5% non-fat milk in TBST). After rinsing in TBST, the primary antibody was laid on overnight. Filters were washed with TBST and PBS. A rabbit anti-mouse horseradish peroxidase conjugated secondary antibody (Sigma) was then laid on at a 1/10,000 dilution for 2 h and after washing, the signal was detected using enhanced chemiluminescence (ECL) (Amersham). **b**, Whole mouse brain homogenates (10%) were prepared in PBS. NP-40 and Na deoxycholate (both from Sigma) were added to a final concentration of 0.25% and the samples were centrifuged at 1000g for 5 min. Half of the supernatant was treated with proteinase K at 50 $\mu\text{g ml}^{-1}$ for 60 min and the reaction terminated with 1 mM Pefabloc (Boehringer). SDS loading buffer (0.25 M Tris-Cl, 8% SDS, 40% v/v glycerol, 0.04% Bromophenol blue, pH 6.8) (4 $\times$) was added and the samples boiled for 10 min before loading. SDS-PAGE gels (12%) were cast and electrophoresed according to previous methods²⁷. Gels were blotted onto Immobilon-P (Millipore), rinsed briefly in PBST (0.05% Tween 20 in PBS), blocked in 5% 'blotto' for 60 min, rinsed in PBST, and then incubated overnight with monoclonal antibody 3F4 at a dilution of 1:5,000 in PBST. Blots were washed in PBST and a rabbit anti-mouse horseradish peroxidase conjugated secondary antibody (Sigma) was laid on at a 1/10,000 dilution in PBST for 60 min. After washing in PBST, blots were developed using ECL on to ECL hyperfilm (Amersham).

mitted several other human prion disease cases to these mice at longer incubation periods, including two inherited prion diseases that failed to transmit to primates^{7,8} (fatal familial insomnia⁹, and inherited prion disease with a 144-base pair insertional mutation in *PRNP* (manuscript in preparation)). The sensitivity of mice expressing both human and mouse PrP, and mice expressing only human PrP (produced by breeding with PrP null mice¹⁰), to human prion diseases are being examined in large-scale transmission experiments. These should determine the rela-

TABLE 1 Primary transmission and passage of CJD to transgenic mice

(a) Incubation periods for different genotypes			
Mouse genotype	Number affected/inoculated	Incubation period (days)	
HuPrP ^{+/-} PrP-p ^{-/-}	8/9	297 ± 15	
HuPrP ^{+/-} Prn-p ^{-/-}	10/10	244 ± 9	
HuPrP ^{+/-} Prn-p ^{o/o}	9/9	212 ± 4	
HuPrP ^{-/+} Prn-p ^{o/o}	8/9	196 ± 3	
HuPrP ^{o/o} Prn-p ^{-/-} (wild type)	0/10	>490	
(b) Type of prion produced			
Inoculum	Recipient mouse genotype	Number affected/inoculated	Incubation period (days)
Primary (CJD case PDG170)	HuPrP ^{-/-} Prn-p ^{o/o}	9/9	212 ± 4
Primary (CJD case PDG170)	HuPrP ^{o/o} Prn-p ^{-/-} (wild type)	0/10	>490
Second passage	HuPrP ^{-/-} Prn-p ^{o/o}	9/9	213 ± 5
Second passage	HuPrP ^{o/o} Prn-p ^{-/-} (wild type)	0/10	>490

a, Incubation periods in transgenic (Tg152) and wild-type mice following inoculation with CJD case PDG170 (mean $\pm$ s.e.m.). Numbers of mice developing illness from the total numbers inoculated are given, losses from intercurrent illness are excluded. $\text{HuPrP}^{+/+}$ and $\text{HuPrP}^{+/0}$ designate mice which were homozygous or hemizygous for the human PrP (Tg152) transgene array, respectively; $\text{Prn-p}^{0/0}$ indicates that the mice were homozygous for PrP null alleles¹⁰; $\text{Prn-p}^{-/-}$ indicates wild type for mouse PrP. Clinical features in these mice were typical of murine scrapie. Neuropathological examination confirmed spongy degeneration of the grey matter, astrocytosis, neuronal loss, and positive PrP immunohistochemistry; age-matched transgenic mice inoculated with saline were histologically normal with negative PrP immunohistochemistry. **b**, Determination of the type of prions produced in mice expressing both human and mouse PrP following challenge with CJD. Mice were inoculated with 1% brain homogenates of transgenic mice of genotype Tg152 $\text{HuPrP}^{+/+} \text{Prn-p}^{-/-}$ that had developed illness following inoculation with CJD case PDG 170. Infectivity generated in these mice is pathogenic for mice expressing only human PrP (genotype Tg152 $\text{HuPrP}^{+/0} \text{Prn-p}^{0/0}$) but not to wild-type mice at 480 days. Incubation periods on primary transmission of human CJD case PDG170 are identical to those on second passage in mice expressing only human PrP (genotype Tg152 $\text{HuPrP}^{+/0} \text{Prn-p}^{0/0}$). Numbers of mice developing illness from the total numbers inoculated are given, losses from intercurrent illness are excluded. Histological examination and immunoblotting studies of these sick mice confirm spongiform neurodegeneration and the presence of human PrP^{Sc} , respectively (data not shown). Strict biosafety protocols were followed. Transgenic mice expressing human PrP were bred and maintained in an animal microbiological containment level I facility and moved to a containment level II facility where intracerebral inoculation was performed in a class I microbiological safety cabinet. Preparation of inocula and removal of tissues were performed in a microbiological containment level III facility. All mice were examined twice each week for the development of clinical signs of scrapie, from which point they were examined daily. Mice were killed if they exhibited any signs of distress. Criteria for clinical diagnosis of scrapie in mice were as described previously²⁴. The Tg152 founder was produced on an FVB background; the Tg110 founder was produced on a C57BL/6 $\times$ 129/Sv background and subsequently crossed with C57BL/6 mice⁵. All mice were genotyped to confirm the presence of the HuPrP transgene or PrP null alleles by polymerase chain reaction (PCR) with genomic DNA obtained by tail biopsy⁶. Tg110 and Tg152 mice have copy numbers of a transgene encoding human PrP of 2–4 and 30–50, respectively⁵; expression levels of human PrP in these lines are 50% and 200% of that seen in normal human brain, respectively⁶. Mice were anaesthetized with halothane/ O_2 and intracerebrally inoculated into the right parietal lobe with 30 μl of a 1% brain homogenate in PBS.

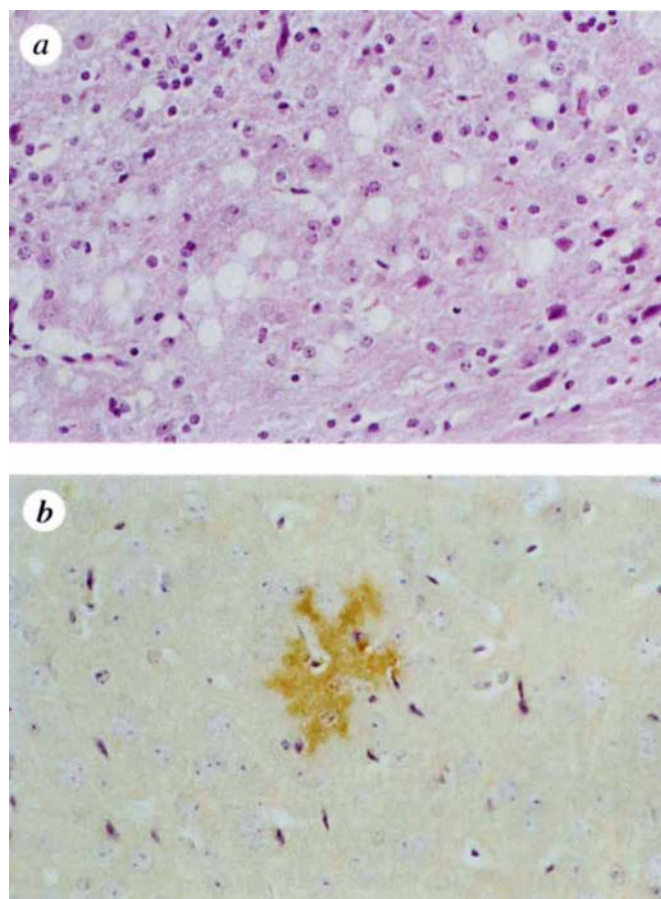
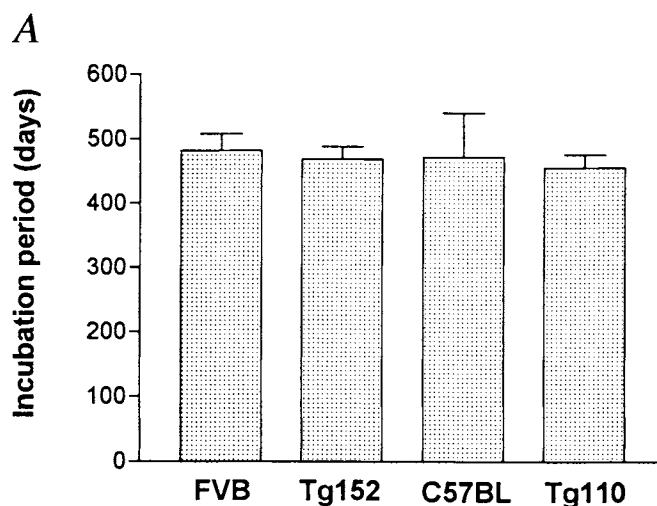
tive utility of such mice compared to other types of laboratory animal for transmission studies of the complete range of human prion diseases (which differ widely in infective titre and regional distribution of PrP^{Sc}), and to determine whether multiple strains of human prions, with different transmission characteristics, exist. In preliminary work it is already clear that mice expressing only human PrP have considerably shorter incubation periods to the same inocula, with 7 of 8 CJD cases transmitting at incubation periods of around 200 days (data not shown).

Incubation period to PDG170 (mean $\pm$ s.e.m.) in Tg110 mice was 484 $\pm$ 40 days (affected/inoculated: 5/6) and in Tg152 mice was 287 $\pm$ 12 days (affected/inoculated: 10/10). The parents of these mice were both transgenic, so some mice will have been homozygous for the transgene array. We repeated these studies in Tg152 mice of various genotypes to study the effect of transgene dosage and removal of mouse PrP (Table 1a). These results

FIG. 2 Transmission of BSE to transgenic and wild-type mice. **A**, Incubation periods (days) in transgenic mice expressing both human and mouse PrP (Tg152 and Tg110) and non-transgenic (FVB and C57BL/6) mice (mean $\pm$ s.e.m.) following inoculation with BSE: FVB wild type, 483 ± 26 ; Tg152, 470 ± 19 ; C57BL/6 wild type, 471 ± 69 ; Tg110, 456 ± 21 . Numbers affected/inoculated were as follows (mice dying after intracerebral inoculation or later from intercurrent illness are excluded): FVB wild type, 13/13; Tg152, 14/15; C57BL/6 wild type, 4/7; Tg110, 11/11. Incubation times for primary transmission of BSE to the C57BL/6 wild-type mice were consistent with those published previously¹⁶; incubation periods in FVB wild-type mice have not been reported previously. Incubation periods did not differ significantly between transgenic mice and the appropriate wild-type control group (Tg152 versus FVB, $P=0.69$; Tg110 versus C57BL/6, $P=0.77$ (unpaired *t*-test; two-tailed)). Clinical features were typical of murine scrapie. Neuropathological studies were performed on mice from all these groups, and confirmed clinical diagnosis. **Ba**, Severe vacuole formation in the medulla oblongata of BSE-inoculated Tg152 mouse. Haematoxylin and eosin, original magnification $\times 100$. **Bb**, PrP-positive deposit in the temporal lobe of BSE-inoculated Tg152 mouse. Avidin biotin complex method, original magnification $\times 100$. The most striking histological feature was the presence of severe spongiform change affecting the grey matter of the brainstem. Vacuoles of various sizes, occasionally coalescing to form microcysts, were seen in the neuropil and sometimes in the neuronal cytoplasm. The mesencephalon, medulla oblongata and diencephalon showed the most severe changes, but the pons, hippocampus, thalamus and corpus striatum were also affected, with an increase in astrocytic nuclei. No scrapie developed in PBS-inoculated control groups, and neither clinical signs of scrapie or histological evidence of spongiform encephalopathy have been seen in PBS inoculated or uninoculated mice from both transgenic lines considerably older (>600 days) than those studied here, excluding spontaneous neuromuscular degeneration, seen in some transgenic lines expressing very high levels of PrP^{Sc}, as a confounding factor in these transmissions.

METHODS. As Table 1. BSE inoculum was prepared from pooled brainstems from neuropathologically confirmed cases of BSE. The presence of PrP^{Sc} in this homogenate was confirmed by immunoblot analysis (data not shown). The titre of agent in the pooled BSE brainstem homogenate is being formally determined, but is likely from previous studies to be in the range 10^3 – 10^5 mouse LD₅₀ (50% lethal dose) per g of brain^{4,29}. Both parents of mice were usually transgenic, so some mice of both Tg110 and Tg152 groups will have been homozygous for the transgene array. When these studies were performed no mouse scrapie had been used in our experimental facility, excluding low-level contamination with mouse scrapie as a cause of some of these transmissions. These studies were performed in a dedicated room not previously used for prion transmissions. No human prions were allowed in this room at any time. **B**, Mice were killed by CO₂ asphyxiation and the brains rapidly removed and fixed using 10% buffered formal saline or were frozen for immunoblot analysis. Fixed tissues were processed according to standard methods, and paraffin-embedded sections stained with haematoxylin and eosin. PrP immunohistochemistry was performed using the avidin biotin complex method and polyclonal antiserum SP40 (ref. 30).

are consistent with similar studies using mice transgenic for Syrian hamster PrP: incubation times are inversely proportional to transgene copy number, and fall further in mice not expressing mouse PrP^{3,11,12}. Immunodotblots demonstrated human PrP^{Sc} in CJD-inoculated sick transgenic mice (Fig. 1a), confirmed on western blotting (Fig. 1b). Saline-inoculated control transgenic mice of the same age were negative. Similar results were obtained with Tg110 mice (data not shown). The type of infectivity produced in these mice was determined by inoculating transgenic mice expressing only human PrP (Tg152 mice of genotype HuPrP^{+/o} Prn-p^{o/o}) and wild-type mice with homogenates from CJD-inoculated transgenic mice (Table 1b). Successful passage to HuPrP^{+/o} Prn-p^{o/o} mice but not to HuPrP^{o/o} Prn-p^{+/+} (wild-type) mice is consistent with the production of human, but not murine, prions in these mice. Incubation periods on primary and second passage with this CJD case were essentially identical, suggesting that HuPrP^{+/o} Prn-p^{o/o} mice lack a species barrier to human prions (Table 1b).



The demonstration that a neurophysiological phenotype in PrP null mice¹³ is rescued by expression of human PrP from the Tg152 transgene array⁶ confirms that human PrP is functional in mouse brain. Mice transgenic for human PrP can produce human PrP^{Sc}, and human-specific infectivity, on challenge with CJD, so the ability of BSE inocula to induce production of human prions can be studied in these mice. We first inoculated mice expressing both human and mouse PrP. Tg152, Tg110 and non-transgenic FVB and C57BL/6 mice were inoculated with BSE, and age-matched control groups were inoculated with just phosphate-buffered saline (PBS). Incubation periods were not significantly different between Tg152 and FVB, and Tg110 and C57BL/6 mice (Fig. 2A). The neuropathological changes in both

transgenic and wild-type mice were similar to those described in both natural and experimentally transmitted BSE¹⁴ (Fig. 2B).

As incubation times are inversely proportional to PrP expression levels³, the failure of human PrP to shorten incubation times, even when expressed at high levels, suggested that no pathological bovine PrP^{Sc}/human PrP^C interactions were occurring, and that these mice died as a result of production of mouse PrP^{Sc} triggered by bovine prions. To investigate this further, we immunoblotted brain homogenates from these mice to determine whether human or mouse PrP^{Sc} had been produced. Human PrP^{Sc} was not detected (Fig. 3), suggesting that these mice produced only mouse prions in response to BSE challenge, rather than human prions or a mixture of the two. Serial dilutions of CJD-inoculated Tg152 mouse brain homogenate with PrP null (*Prn-p^{0/0}*) mouse brain homogenate were immunoblotted using monoclonal antiserum 3F4. Human PrP^{Sc} was detected at a 1 in 300 dilution (data not shown). Passage of BSE-inoculated mouse brain homogenates into both wild-type mice and mice expressing only human PrP are in progress to determine whether

low levels of human prions, not detected by immunoblotting, were produced.

Wild-type mice have been shown to be susceptible to BSE^{15–17}. We challenged these transgenic mice with BSE by intracerebral inoculation, a route approximately 10⁵-fold more efficient than oral exposure in mice¹⁸ (the large majority of human BSE exposure will have been oral). Challenging mice that produce both mouse PrP and high levels of human PrP appears to result only in production of mouse PrP^{Sc}, which suggests that human PrP^C is less susceptible than mouse PrP^C to conversion to PrP^{Sc} by bovine prions. However, it is possible that conversion of human PrP^C to PrP^{Sc} occurs less efficiently in mouse cells than conversion of mouse PrP^C, although the short incubation periods possible on primary transmission of CJD to HuPrP^{+/+} *Prn-p^{0/0}* mice indicates that this effect is unlikely to be large, and may be compensated by overexpression of human PrP. Interference with human prion propagation by mouse PrP may also be relevant, as incubation periods to CJD are reduced in mice expressing only human PrP.

As a second stage in our analysis of the bovine to human species barrier, we inoculated HuPrP^{+/+} *Prn-p^{0/0}* mice with BSE; such mice express only human PrP and can therefore only produce human PrP^{Sc}. Groups of 10 mice were inoculated with the pooled BSE brainstem homogenate used in our previous studies, and also with brainstem homogenates derived from four individual, autopsy-confirmed, cases of BSE. Excluding losses from intercurrent illness, these mice remain well at 264 days post-inoculation. Although this period is too short to draw any firm conclusions, the incubation period is already approximately 60 days longer than that for CJD in mice of this genotype. The end point of this study may still be 600–700 days away if these mice die of old age rather than illness.

The human PrP transgene used in these studies encodes valine at polymorphic residue 129, known to be involved in genetic susceptibility to human prion diseases^{19, 21}. Human individuals homozygous for PrP valine 129 appear to be the most susceptible genotype following exposure to human prions^{20, 21}. However, it will be important to repeat these studies with mice expressing human PrP methionine 129 and with transgenic 'heterozygotes', to determine whether heterozygosity at codon 129 protects against the transmission of BSE, as it does against the development or rate of progression of acquired, sporadic and some types of inherited human prion diseases^{19, 20, 22, 23}.

Although these studies using transgenic mice provide an opportunity to study whether bovine prions can induce formation of human PrP^{Sc}, it is important to note that many other factors may be relevant in determining human susceptibility to BSE. □

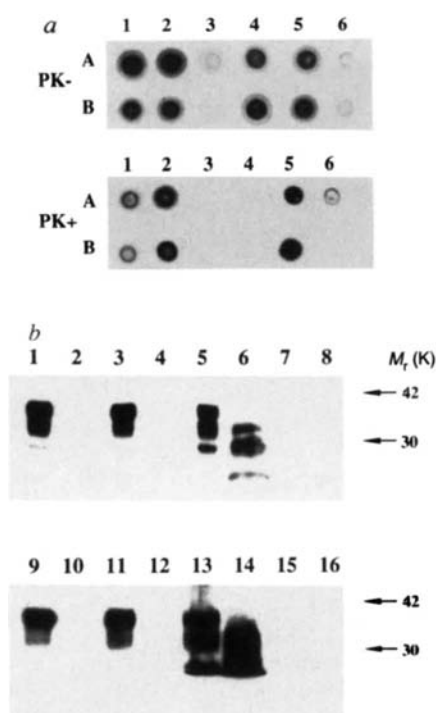


FIG. 3 Determination of the species type of PrP^{Sc} produced in transgenic mice expressing both human and mouse PrP, following inoculation with BSE. *a*, Immunodotblot of brain homogenates using anti-PrP polyclonal R073, which detects both human and mouse PrP^{Sc}. A1, BSE-inoculated Tg152 mouse; A2, BSE-inoculated FVB (wild-type) mouse; A3, saline-inoculated Tg152 mouse; A4, saline-inoculated FVB (wild-type) mouse; A5, CJD-inoculated Tg152 mouse; A6, human brain from CJD case; B1, BSE-inoculated Tg110 mouse; B2, BSE-inoculated C57BL/6 (wild-type) mouse; B3, saline-inoculated Tg110 mouse; B4, saline-inoculated C57BL/6 (wild-type) mouse; B5, CJD-inoculated Tg110 mouse; B6, normal human brain. PK+ and PK- indicate with or without pretreatment with proteinase K, respectively. *b*, Western blots of brain homogenates using monoclonal antibody 3F4, which detects human but not mouse PrP^{Sc}. Odd-numbered lanes were not treated with proteinase K (PK); even-numbered lanes were treated with PK. Lanes: 1 and 2, BSE-inoculated Tg152 mouse; 3 and 4, saline-inoculated Tg152 mouse; 5 and 6, CJD-inoculated Tg152 mouse; 7 and 8, BSE-inoculated FVB (wild-type) mouse; 9 and 10, BSE-inoculated Tg110 mouse; 11 and 12, saline-inoculated Tg110 mouse; 13 and 14, CJD-inoculated Tg110 mouse; 15 and 16, BSE-inoculated C57BL/6 (wild-type) mouse.

METHODS. *a*, As Fig. 1a, except that a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Sigma) was used. *b*, As for Fig. 1b.

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Diurnal variation in mRNA encoding serotonin *N*-acetyltransferase in pineal gland

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FORMATION of the pineal gland hormone melatonin increases markedly at night in response to light–dark environmental alterations^{1–5}. Melatonin is synthesized from serotonin by an initial *N*-acetylation followed by methylation of the 5-hydroxy moiety by hydroxyindole-*O*-methyltransferase^{6,7}. Serotonin *N*-acetyltransferase (NAT; EC2.3.1.87), which catalyses the first reaction, is the rate-limiting enzyme in this process, and its activity increases dramatically with the onset of darkness. Because melatonin may play important biological roles in reproduction^{1,2,8}, ageing^{1,2,9} and sleep^{1,2,9}, understanding the molecular factors that regulate NAT is of particular importance. To identify proteins that regulate

light–dark variations in pineal function, we used a subtractive hybridization technique based on the polymerase chain reaction (PCR) to isolate rat pineal gland messages that are differentially expressed by day and night. Here we report the molecular cloning of NAT and dramatic diurnal variations in its transcription. Independently, Klein and associates have cloned NAT from sheep pineal glands¹⁰.

Conventional subtractive hybridization procedures require large amounts of tissue. Because the rat pineal gland is so small (only 1 mg), we applied a technique¹¹ in which PCR is used to amplify small amounts of complementary DNA in successive rounds of subtraction. We enriched night (2:00)-specific pineal cDNA by successive rounds of subtraction with daytime (14:00) cDNA, followed by PCR amplification. From this subtracted cDNA pool, we cloned a night-enriched cDNA fragment (PF12) and used it as a probe to study its expression. This probe revealed a transcript expressed abundantly in the pineal at night but absent during the day (Fig. 1a). A single robust band of 1.6 kilobases (kb) was detected by northern analysis in the pineal gland with very low levels in eye and testis, and no detectable expression in any other tissue examined (Fig. 1b).

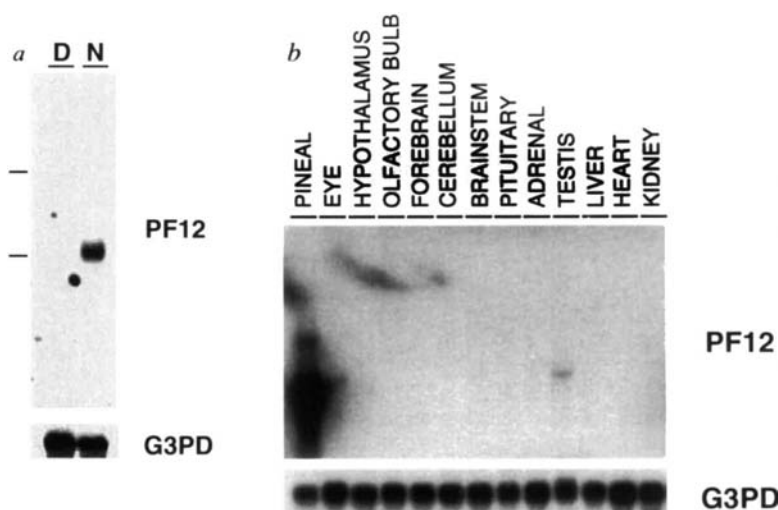
Analysis of pineal messenger RNA from rats that were entrained at light: dark (LD) 14:10 (with lights on at 7:00) at different time points indicated a sudden increase in expression of this gene between midnight and 2:00 (3 and 5 hours after the onset of darkness), and an abrupt decline from its peak level at 6:00 to undetectable values at 8:00, one hour after the start of the light period (Fig. 2a). This pattern is identical to that of pineal NAT activity measured in parallel experiments (Fig. 2b).

The pineal-specific nature of the transcript and its temporal expression pattern suggested that it might encode NAT. We constructed a cDNA library from pineal glands of 200 rats killed at 2:00, and screened the library with the PF12. We identified a cDNA of 1.6 kb which encodes a protein comprising 205 amino acids (Fig. 3). *In vitro* transcription and translation of the cDNA resulted in a protein of the expected relative molecular mass (M_r , 23K). A GenBank search revealed no close homology with any known mammalian protein. The sequence possesses several potential sites for phosphorylation by protein kinase A (PKA), protein kinase C (PKC) and casein kinase.

We expressed this cDNA by transfecting HEK 293 cells, and assayed resultant cell extracts for NAT activity (Table 1). Only negligible NAT activity was evident in mock-transfected cells, whereas substantial activity occurred with the transfected cell

FIG. 1 The PCR-based subtractive hybridization technique identifies a single transcript detected primarily in the night pineal gland. **a**, Northern blot analysis of total pineal RNA (5 µg) from rats at 14:00 (D) or 2:00 (N) probed with PCR fragment PF12. Equal loading of the RNA samples was confirmed by probing an identical blot with G3PD. **b**, Northern blot analysis of a panel of rat tissue total RNAs (5 µg each) at night (2:00) with PF12 as probe. Equal loading and quality of RNAs were confirmed by G3PD controls. No signal was detected in the same set of tissues from daytime (14:00) rats (data not shown). The band of higher M_r seen in the pineal lane was not reproducible.

METHODS. All animals used in this work were adult male Sprague Dawley rats obtained from Sasco. Rats were housed in LD 14:10, with lights off at 21:00, for at least one week before experiments. Daytime animals were killed at 14:00, night-time animals were killed in the dark under safelight at 2:00. The mRNAs were isolated from 400 pineal glands of the day and night rats using standard techniques²². *Alu1*-digested, and linker-ligated, double-stranded cDNAs prepared from day and night pineal mRNAs were amplified by PCR for the subsequent subtractive hybridization. PCR-based subtractive hybridization was done as described¹¹. After three cycles of subtractive enrichment, the PCR products were subcloned into a plasmid vector. One of the fragments (PF12, 150 base pairs) was specifically enriched in the PCR-



amplified night pineal cDNA population, and was used to probe the day/night pineal northern blot as well as the day/night rat tissue RNA blots. RNA extraction and northern analysis were done as described²².

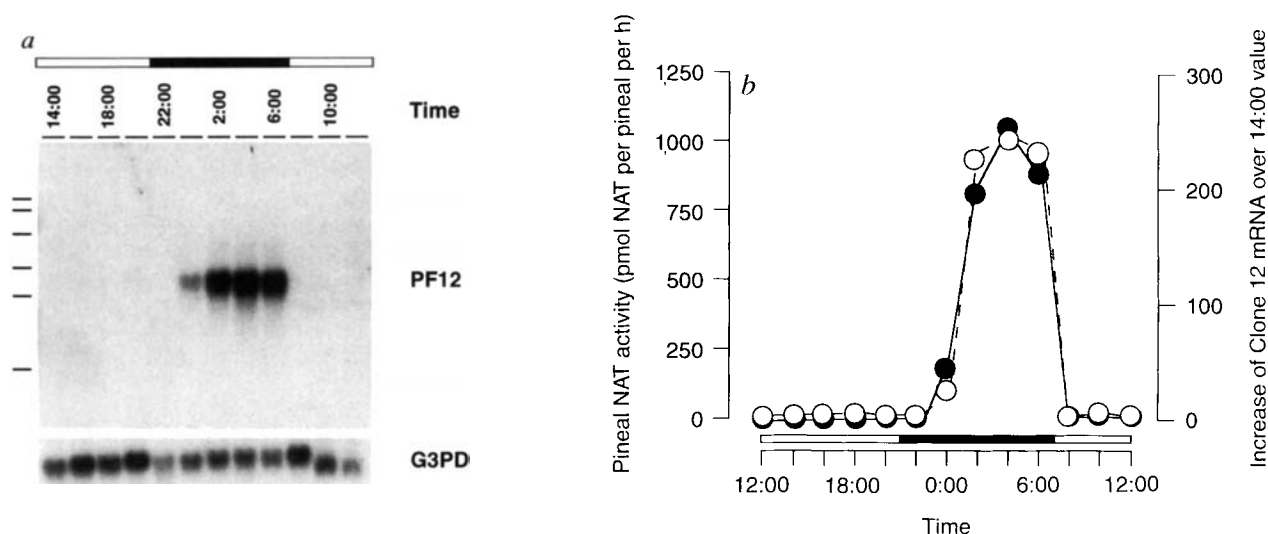


FIG. 2 The temporal expression pattern of PF12 mRNA overlaps with that of pineal NAT activity. **a**, Northern blot analysis using probe PF12 of pineal mRNAs at the times indicated. Horizontal bars indicate RNA size standards of 9.49, 7.46, 4.40, 2.37, 1.35 and 0.24 kb (from top to bottom). **b**, Day and night studies of rat pineal NAT activity and PF12 mRNA analysis.

METHODS. Rats housed in LD 14:10 for more than a week were killed at 2-h intervals over a 24-h period. During the night period (21:00–7:00), rats were killed under safelight. At each time point, nine pineal glands were collected for mRNA preparations, and three glands for NAT assay. The mRNA samples shown in **a** were the equivalent of 1.5 pineal glands each. The northern blots in **a** were analysed using a Phosphor-imager program (Molecular Dynamics). Data were normalized to G3PD mRNA level for each time point, and are expressed relative to values

at 14:00. The pineal NAT assay was modified from a previous assay²³. Three glands at each time point were homogenized by sonication in 150 μ l of 0.1 M phosphate buffer, pH 6.8. Portions of 1 μ l (1/50 of a pineal gland) were incubated in the presence of 1 μ l tryptamine-HCl (20 mM), 1 μ l acetyl-(1-¹⁴C) coenzyme A (NEN, 60 mCi mmol⁻¹, 20 nCi per sample) in a total volume of 10 μ l. Incubation at 37 °C for 30 min was stopped by the addition of 200 μ l of 0.1 M sodium borate buffer, pH 10. The *N*-acetyl-(¹⁴C) tryptamine formed was extracted once with 1 ml chloroform and the aqueous phase removed. The organic phase was rinsed once with the borate buffer. A 0.5-ml portion of each chloroform extract was allowed to dry and radioactivity was quantified. The data presented have been corrected for background in the absence of homogenate and background in the absence of substrate.

extracts using the known NAT substrates¹² tryptamine, 5-methoxytryptamine and 5-hydroxytryptamine (serotonin). We also examined aniline, a substrate for arylamine *N*-acetyltransferase^{13,14}, which is distinct from serotonin NAT. There was no detectable enhancement of acetylation of aniline by transfected cell extracts. Based on the expression pattern and the substrate specificity of this cDNA, we conclude that we have cloned the pineal NAT.

The extraordinary diurnal variation in pineal NAT activity indicates that this enzyme may be one of the most tightly regulated of all proteins. Its cloning provides an approach to studying the molecular basis of its regulation.

The pineal gland of the rat receives its primary innervation from sympathetic neurons of the superior cervical ganglion^{15,16}. NAT activity correlates with norepinephrine release from these fibres, which increases the level of pineal cyclic AMP¹⁷. Thus it seems likely that NAT transcription is modulated by cAMP response element (CRE)-binding factors. The sudden decline in NAT mRNA, which parallels the drop in NAT activity, indicates that termination of NAT transcription primarily underlies the abrupt decrease in enzyme activity. An alternatively spliced, pineal-enriched form of CRE-binding factor, inducible cAMP early repressor (ICER), is known to vary diurnally^{18,19}. ICER inhibits cAMP-induced transcription through CRE sites^{20,21}. It is likely that ICER is essential for downregulation of NAT transcription. There is no detectable time delay between the decline of NAT activity and the decrease in NAT transcript level, which suggests that the enzyme is inactivated rapidly by post-translational modification and/or proteolysis. This might also be accomplished through cAMP by phosphorylation of the multiple PKA sites in NAT.

Expression cloning has been used¹⁰ independently to identify a sheep pineal NAT cDNA. The sheep and rat pineal NAT sequences display about 75% amino-acid sequence homology,

but there are several differences between sheep and rat NAT: sheep NAT is expressed in pituitary and brainstem, whereas rat NAT is not detectable in these areas; and NAT mRNA levels are 100-fold higher at night than in the day in rat pineal gland, whereas in sheep the night levels are only twice as high as daytime values¹⁰.

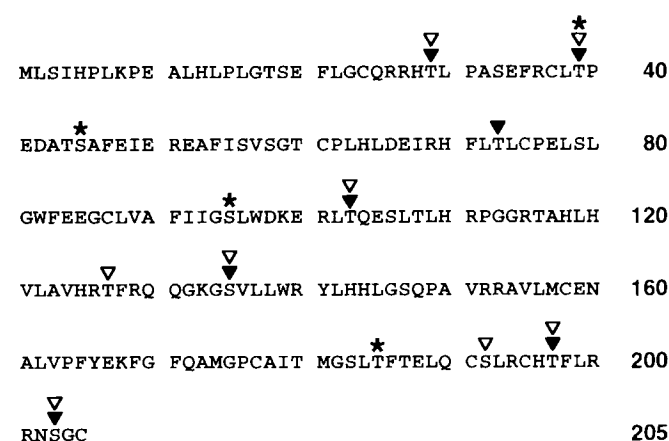


FIG. 3 Amino acid sequence of rat clone 12 (GenBank accession number U40803). Asterisks, putative casein kinase II phosphorylation sites; filled triangles, putative cAMP- and cGMP-dependent protein kinase phosphorylation sites; open triangles, putative PKC phosphorylation sites²⁴. This open reading frame is preceded by an in-frame termination codon.

METHODS. The night-time (2:00) pineal mRNA was used to construct a cDNA library in λ -ZIPLOX (GIBCO-BRL) according to the manufacturer's protocol. The mRNA preparation and library screening using ³²P-labelled PF12 were performed as described previously²².

TABLE 1 Functional expression of NAT activity

Substrate	Enzyme activity (nmol mg ⁻¹ hr ⁻¹)	
	Mock-transfected	Transfected with clone 12.7
Arylalkylamines		
Tryptamine	0.6	1256
5-Methoxytryptamine	0.9	1232
5-Hydroxytryptamine	1.2	1064
Arylamine		
Aniline	2.9	1

The HEK 293 cell extracts were obtained 40 h after transfection with either control DNA or an expression plasmid pCIS12.7. Cells were collected from 10-cm dishes, rinsed twice with PBS buffer, sonicated in 500 µl of 0.1 M phosphate buffer, pH 6.8, and spun at 14,000g for 10 min. The supernatant (1 µl from each sample) was mixed with indicated substrates (final concentration 2 mM) and 1 µl of acetyl-(1-¹⁴C) coenzyme A (60 mCi mmol⁻¹, 20 nCi per sample) in a reaction volume of 10 µl. After incubation at 37 °C for 30 min, the reaction mixture was quenched with 200 µl 0.1 M sodium borate (pH 10) and extracted with 1 ml toluene: isoamyl alcohol (97:3), except for 5-OH-tryptamine, which was extracted with straight isoamyl alcohol¹³. The mixture was vortexed for 1 min and centrifuged for 5 min. A 0.8-ml aliquot of each extracted organic phase was transferred to a scintillation vial and radioactivity was counted.

The cloning of NAT should greatly facilitate studies of melatonin formation, including molecular means of controlling its biosynthesis. This may make possible the development of thera-

peutic agents that might regulate melatonin-sensitive processes, such as biological clock functions, reproduction, ageing and sleep. □

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Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B

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GLYCOGEN synthase kinase-3 (GSK3)¹ is implicated in the regulation of several physiological processes, including the control of glycogen² and protein³ synthesis by insulin, modulation of the transcription factors AP-1 and CREB^{4–6}, the specification of cell fate in *Drosophila*⁷ and dorsoventral patterning in *Xenopus* embryos⁸. GSK3 is inhibited by serine phosphorylation in response to insulin or growth factors^{3,9–11} and *in vitro* by either MAP kinase-activated protein (MAPKAP) kinase-1 (also known as p90^{rk}) or p70 ribosomal S6 kinase (p70^{S6k})^{12,13}. Here we show, however, that agents which prevent the activation of both MAPKAP kinase-1 and p70^{S6k} by insulin *in vivo* do not block the phosphorylation and inhibition of GSK3. Another insulin-stimulated protein kinase inactivates GSK3 under these conditions, and we demonstrate that it is the product of the proto-oncogene protein kinase B (PKB, also known as Akt/RAC). Like the inhibition of GSK3 (refs 10, 14), the activation of PKB is prevented by inhibitors of phosphatidylinositol (PI) 3-kinase.

The 40–50% inhibition of GSK3 induced by insulin in L6 myotubes (Fig. 1a–c) was unaffected by agents which prevented the activation of MAPKAP kinase-1 (8-bromo-cyclic AMP, or PD 98059 (ref. 15); (Fig. 1d, e) and/or p70^{S6k} (rapamycin¹⁶)

(ref. 10), suggesting that neither MAPKAP kinase-1 nor p70^{S6k} are essential for this process. However, the phosphorylation and inhibition of GSK3-β after phorbol ester treatment¹⁷ is enhanced by coexpression with MAPKAP kinase-1 in HeLa S3 cells, whereas in NIH 3T3 cells the EGF-induced inhibition of GSK3-α and GSK3-β¹¹ is largely suppressed by expression of a dominant-negative mutant of MAP kinase kinase-1 (ref. 18). MAPKAP kinase-1 may therefore mediate the inhibition of GSK3 by agonists which are much more potent activators of the classical MAP kinase pathway than is insulin.

To identify the insulin-stimulated protein kinase that inhibits GSK3 in the presence of rapamycin and PD 98059, L6 myotubes were incubated with both compounds and stimulated with insulin. The lysates were then chromatographed on Mono Q and the fractions assayed with 'Crosstide' (GRPTSSFAEG), a peptide corresponding to the sequence in GSK3 surrounding the serine (underlined) phosphorylated by MAPKAP kinase-1 and p70^{S6k} (Ser 21 in GSK3-α¹³ and Ser 9 in GSK3-β¹²). Three peaks of Crosstide kinase activity were detected, which were absent if insulin stimulation was omitted or if the cells were first preincubated with the PI 3-kinase inhibitor wortmannin (Fig. 2a). Wortmannin^{10,14} and the structurally unrelated PI 3-kinase inhibitor LY 294002 (ref. 19) (Fig. 1b) both prevent the inhibition of GSK3 by insulin.

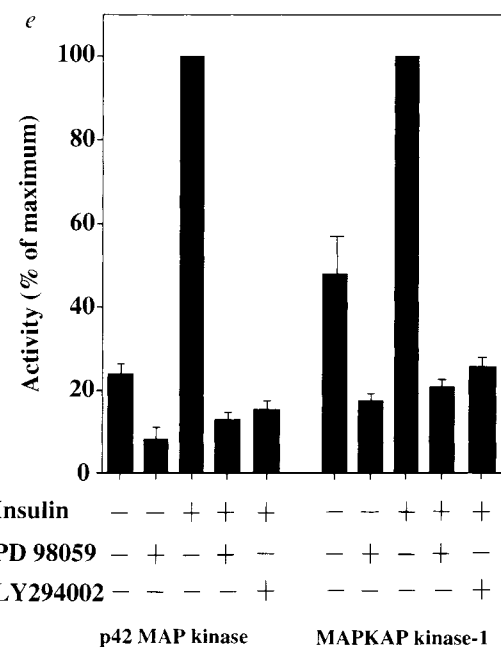
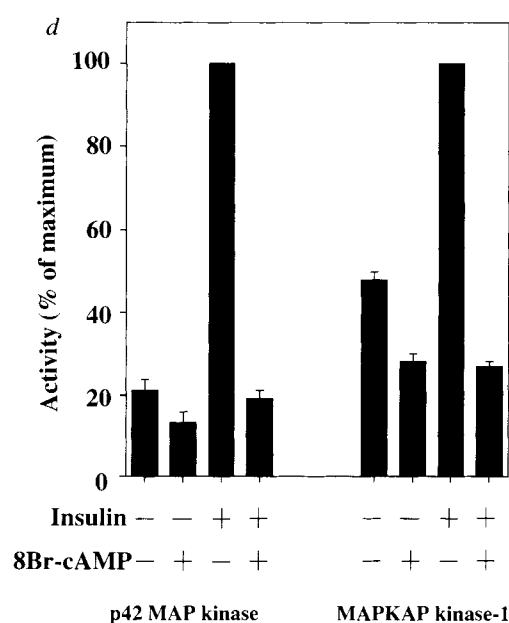
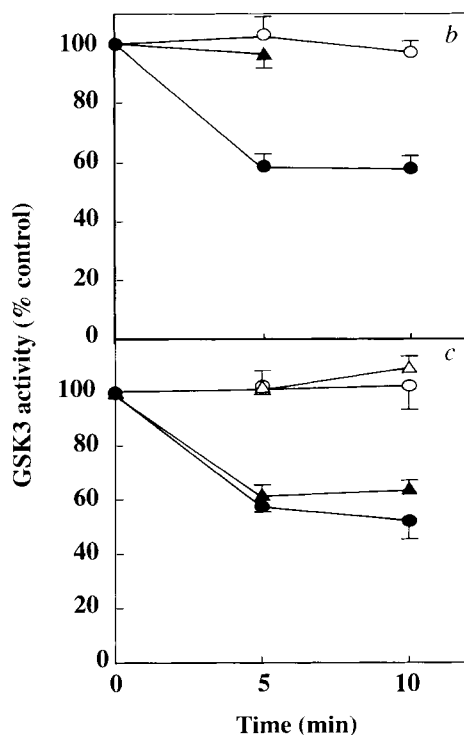
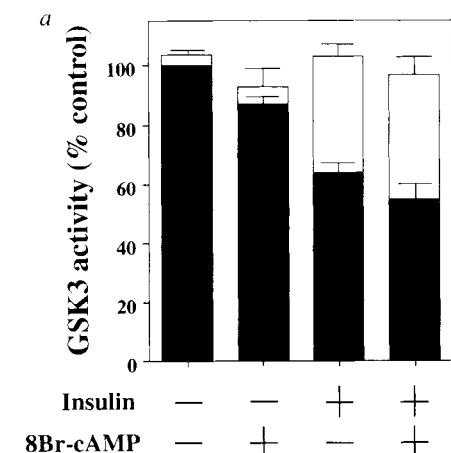
The protein kinases PKB-α (also known as Akt-1/RAC-α) and PKB-β (also known as Akt-2/RAC-β) are Ser/Thr-specific and cellular homologues of the viral oncogene *v-akt*^{20–23}. These enzymes have recently been shown to be activated in NIH 3T3, Rat-1 or Swiss 3T3 cells in response to growth factors or insulin, activation being suppressed by blocking the activation of PI 3-kinase in different ways^{24,25} (M. Andjelkovich *et al.*, manuscript submitted). All three peaks of Crosstide kinase (Fig. 2a), but no other fraction from Mono Q, showed the characteristic multiple bands of PKB (relative molecular mass, *M_r*, 58K, 59K or 60K) that have been observed in other cells, when immunoblotting was performed with an antibody raised against the carboxy-

terminal peptide of PKB- α (Fig. 2b). The more slowly migrating forms represent more highly phosphorylated protein, and are converted to the fastest migrating species by treatment with phosphatases. Phosphatase treatment also results in the inactivation of PKB²⁵ (M. Andjelkovich *et al.*, manuscript submitted) and the complete loss of Crosstide kinase activity (data not shown). Of the Crosstide kinase activity in peaks 2 and 3 from Mono Q, 70–80% was immunoprecipitated by a separate antibody raised against the amino-terminal pleckstrin homology (PH) domain of PKB- α . The C-terminal antibody also immunoprecipitated PKB activity specifically from peaks 2 and 3, but

was less effective than the anti-PH-domain antibody. Peak-1 was hardly immunoprecipitated by either antibody and may represent PKB- β . An immunoprecipitating anti-MAPKAP kinase-1 antibody¹⁰ failed to deplete any of the Crosstide kinase activity associated with peaks 1, 2 or 3.

Insulin stimulation of L6 myotubes increased PKB activity by more than tenfold (Fig. 2c), and activation was blocked by wortmannin or LY 294002, but was essentially unaffected by 8-bromo-cyclic AMP or rapamycin plus PD 98059 (Fig. 2c). The half-time ($t_{0.5}$) for activation of PKB (1 min) was slightly faster than that for inhibition of GSK3 (2 min)¹⁰. In contrast, the

FIG. 1 The inhibition of GSK3 by insulin in L6 myotubes is unaffected by agents that prevent activation of the classical MAP kinase pathway. All the results ($\pm$ s.e.m.) are for at least three experiments. *a*, L6 myotubes were incubated for 15 min with 2 mM 8-bromocyclic-AMP (8Br-cAMP) and then with 0.1 μ M insulin (5 min). Both GSK3 isoforms were co-immunoprecipitated from the lysates and assayed before (black bars) and after (white bars) reactivation with PP2A₁¹⁰. The results are presented relative to the activity in unstimulated cells, which was 0.08 ± 0.006 U mg⁻¹ ($n=10$).



b, *c*, The inhibition of GSK3 by insulin (0.1 μ M) is unaffected by rapamycin (0.1 μ M) and PD 98059 (50 μ M), but prevented by LY 294002 (100 μ M). *b*, L6 myotubes were stimulated with insulin for the times indicated with (filled triangle) or without (filled circles) a 15-min preincubation with LY 294002, and GSK3 measured as in *a*. The open circles show experiments from insulin-stimulated cells where GSK3 was assayed after reactivation with PP2A₁¹⁰. In *c*, cells were incubated with rapamycin (triangles) or rapamycin plus PD 98059 (circles) before stimulation with insulin, and GSK3 activity measured as in *a*, before (filled symbols) and after (open symbols) pretreatment with PP2A. *d*, *e*, L6 myotubes were incubated with 8Br-cAMP (15 min), PD 98059 (60 min) or LY 294002 (15 min) and then with insulin (5 min) as in *a*–*c*. Each enzyme was assayed after immunoprecipitation from lysates, and the results are presented relative to the activities obtained in the presence of insulin and absence of 8Br-cAMP, which were 0.04 ± 0.005 U mg⁻¹ (p42 MAP kinase, $n=6$) and 0.071 ± 0.004 U mg⁻¹ (MAPKAP kinase-1, $n=6$).

METHODS. Monolayers of L6 cells were cultured until myotubes had formed, stimulated and lysed as described previously¹⁰. p42 MAP kinase, MAPKAP kinase 1 or (GSK3- α plus GSK3- β) were then immunoprecipitated from the lysates and assayed with specific protein or peptide

substrates as described previously¹⁰. One unit of protein kinase activity was that amount which catalysed the phosphorylation of 1 nmol of substrate in 1 min. Where indicated, GSK3 in immunoprecipitates was reactivated with PP2A₁¹⁰.

activation of MAPKAP kinase-1 (Fig. 2d) and p70^{S6k} (not shown) was slower ($t_{0.5} > 5$ min). Activation of MAPKAP kinase-1 was prevented by 8-bromo-cyclic AMP or PD 98059 (Fig. 2d), and activation of p70^{S6k} by rapamycin¹⁰. PKB phosphorylated the Ser in Crosstide equivalent to Ser 21 in GSK3- α and Ser 9 in GSK3- β (data not shown).

PKB from insulin-stimulated L6 myotubes (but not from unstimulated or wortmannin-treated cells) inactivated GSK3- α and GSK3- β *in vitro*, and inhibition was reversed by the Ser/Thr-specific protein phosphatase PP2A₁ (Fig. 3a). To further establish that inactivation was catalysed by PKB, and not by a co-immunoprecipitating protein kinase, haemagglutinin-tagged

PKB- α (HA-PKB) was transfected into COS-1 cells and activated by stimulation with pervanadate, which is the strongest inducer of PKB activation in this system (M. Andjelkovich *et al.*, manuscript submitted). The HA-PKB inactivated GSK3- β , but not if treatment with pervanadate was omitted or if wild-type HA-PKB was replaced with a 'kinase inactive' mutant (Fig. 3b).

The inactivation of GSK3- β by PKB *in vitro* was accompanied by the phosphorylation of one major tryptic peptide (Fig. 4a) which coeluted during C₁₈ chromatography¹² and isoelectric focusing with that obtained after phosphorylation by MAPKAP kinase-1 (Fig. 4d). Stimulation of L6 myotubes with insulin (in

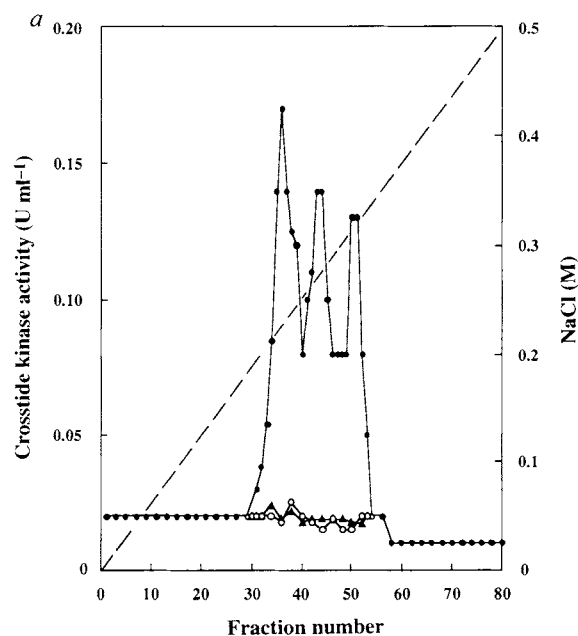
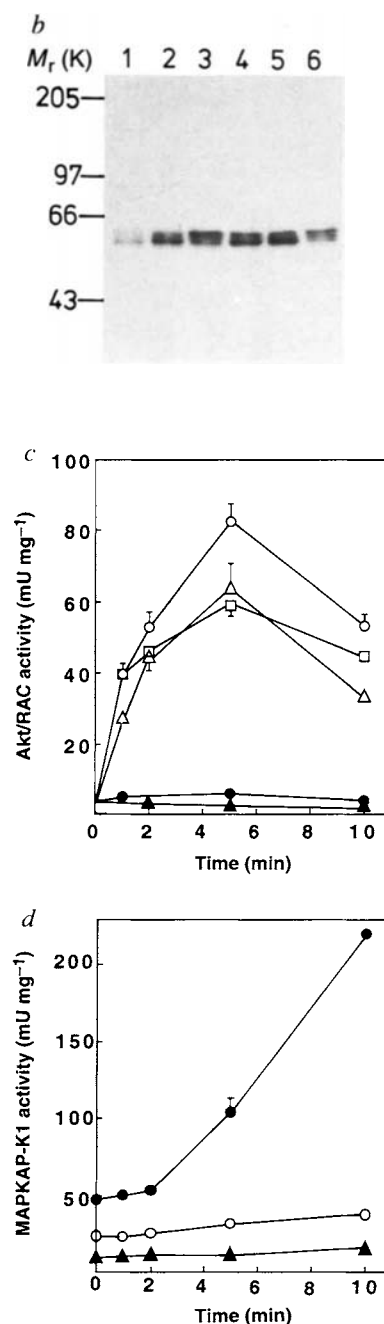


FIG. 2 Identification of PKB as the insulin-stimulated, wortmannin-sensitive and PD 98059/rapamycin-insensitive Crosstide kinase in L6 myotubes. *a*, Cells were incubated with 50 μ M PD 98059 (for 1 h) and 0.1 μ M rapamycin (10 min), then stimulated with 0.1 μ M insulin (5 min) and lysed¹⁰. The lysates (0.3 mg protein) were chromatographed on Mono Q (5 $\times$ 0.16 cm) and fractions (0.05 ml) were assayed for Crosstide kinase (filled circles). In separate experiments insulin was omitted (open circles) or wortmannin (0.1 μ M) added 10 min before the insulin (filled triangles). The broken line shows the NaCl gradient. Similar results were obtained in six experiments. *b*, Pooled fractions (10 μ l) 31–34 (lane 1), 35–38 (lane 2), 39–42 (lane 3), 43–45 (lane 4), 46–49 (lane 5) and 50–53 (lane 6) from *a* were electrophoresed on a 10% SDS/polyacrylamide gel and immunoblotted with the C-terminal anti-PKB- α antibody. Marker proteins are indicated. No immunoreactive species were present in fractions 1–30 or 54–80. *c*, L6 myotubes were stimulated with 0.1 μ M insulin and PKB immunoprecipitated from the lysates (50 μ g protein) essentially as described previously¹⁰, using the anti-PH domain antibody and assayed for Crosstide kinase (open circles). In control experiments, myotubes were incubated with 0.1 μ M rapamycin plus 50 μ M PD 98059 (open triangles) or 2 mM 8Br-cAMP (open squares), or 0.1 μ M wortmannin (filled circles) or 100 μ M LY 294002 (filled triangles) before stimulation with insulin. *d*, As *c*, except that MAPKAP kinase-1 was immunoprecipitated from the lysates and assayed with S6 peptide (filled circles). In control experiments, cells were incubated with 0.1 μ M rapamycin plus 50 μ M PD 98059 (filled triangles) or with 2 mM 8Br-cAMP (open circles) before stimulation with insulin. In *c* and *d*, the error bars denote triplicate determinations, and similar results were obtained in three separate experiments.

METHODS. Mono Q chromatography was performed as described¹³, except that the buffer also contained 1 mM EGTA, 0.1 mM sodium orthovanadate and 0.5% (w/v) Triton X-100. Two anti-PKB- α antibodies were raised in rabbits against the C-terminal peptide FPQFSYSASSTA and bacterially expressed PH domain of PKB- α . The C-terminal antibody



was affinity purified²¹. The activity of PKB towards Crosstide is threefold higher than its activity towards histone H2B and 11-fold higher than its activity towards myelin basic protein, the substrates used previously to assay PKB. Other experimental details and units of protein kinase activity are given in Fig. 1.

the presence of rapamycin and PD 98059) increased the ^{32}P -labelling of GSK3- α and GSK3- β by 60–100% (Fig. 4c) and increased the ^{32}P -labelling of the same tryptic peptides labelled *in vitro* (Fig. 4d). Sequence analyses established that the third residue of these, corresponding to Ser 9 (GSK3- β) or Ser 21

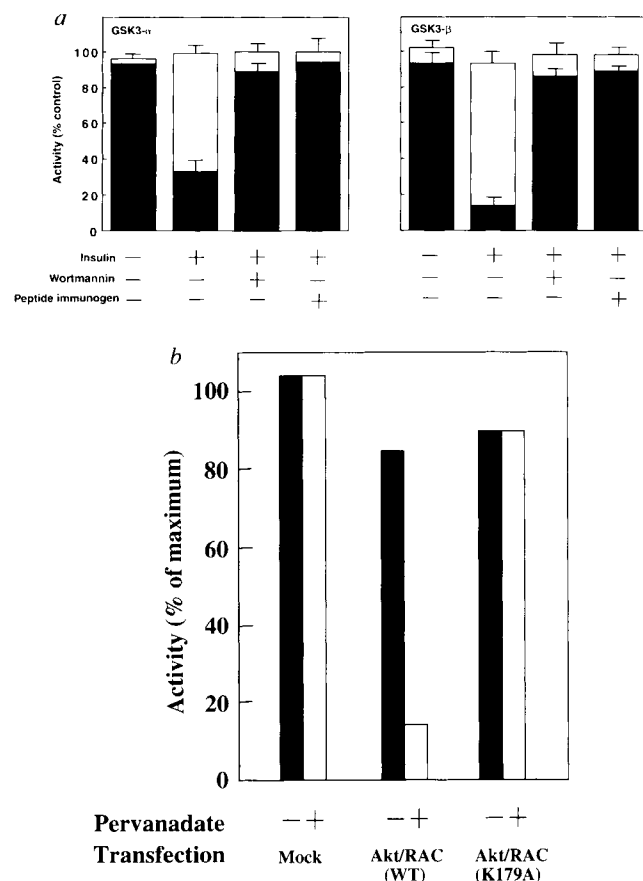


FIG. 3 GSK3 is inactivated by PKB from insulin-stimulated L6 myotubes. **a**, Cells were stimulated for 5 min with 0.1 μM insulin, and PKB immunoprecipitated from 100 μg of cell lysate and used to inactivate GSK3 isoforms essentially as described previously^{12,13}. The black bars show GSK3 activity measured after incubation with MgATP and PKB as a percentage of the activity obtained in control incubations where PKB was omitted. In the absence of PKB, GSK3 activity was stable throughout the experiment. The white bars show the activity obtained after reactivation of GSK3 with PP2A₁. No inactivation of GSK3 occurred if insulin was omitted, or if wortmannin (0.1 μM) was added 10 min before the insulin, or if the anti-PKB antibody was incubated with peptide immunogen (0.5 mM) before immunoprecipitation. The results ($\pm$ s.e.m.) are for three experiments (each carried out in triplicate). **b**, Inactivation of GSK3- β by HA-PKB- α . Complementary DNA encoding HA-PKB- α was transfected into COS-1 cells, and after stimulation for 15 min with 0.1 mM sodium pervanadate (M. Andjelkovich, manuscript submitted) the tagged protein kinase was immunoprecipitated from 0.3 mg of lysate and incubated for 20 min with GSK3- β and MgATP. In control experiments, pervanadate was omitted, or wild-type (WT) PKB- α replaced by vector (mock transfection) or by a kinase-inactive mutant of PKB- α in which Lys 179 was mutated to Ala (K179A). Similar results were obtained in three separate experiments. The levels of WT and K179A PKB- α in each immunoprecipitate were similar in each transfection.

METHODS. In **a**, PKB- α was immunoprecipitated with the C-terminal antibody from lysates (0.5 mg protein) of insulin-stimulated L6 myotubes. GSK3- α and GSK3- β were partially purified, assayed, inactivated by PKB, and reactivated by PP2A₁ from rabbit skeletal muscle as described previously^{12,13}. There was no reactivation in control experiments in which okadaic acid (2 μM) was added before PP2A₁. Other experimental details are described in Figs 1 and 2.

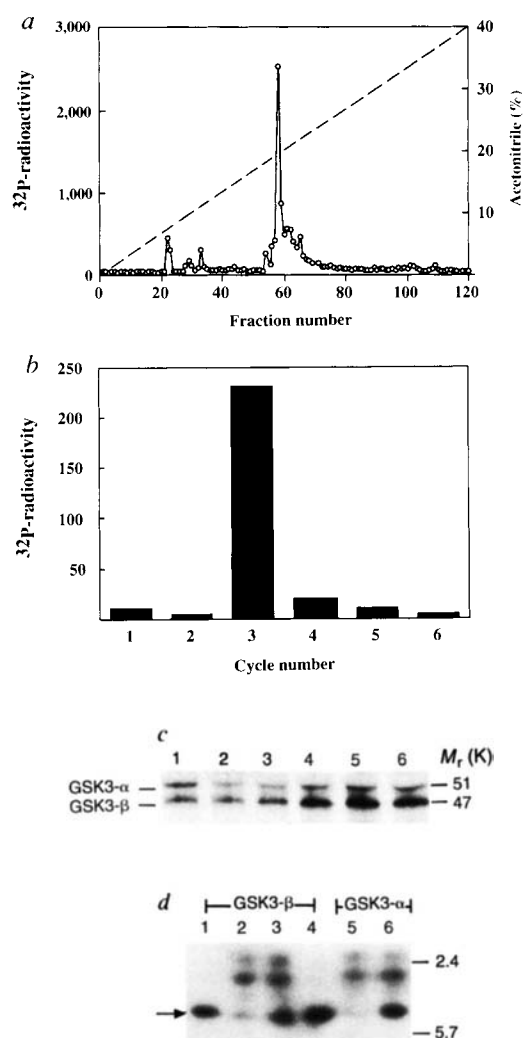


FIG. 4 Identification of the residues in GSK3 phosphorylated by PKB *in vitro* and in response to insulin in L6 myotubes. **a**, GSK3- β was maximally inactivated by incubation with PKB and Mg- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and after SDS-PAGE, the ^{32}P -labelled GSK3- β (M_r 47K) was digested with trypsin¹¹ and chromatographed on a C₁₈ column¹². Fractions (0.8 ml) were analysed for ^{32}P -radioactivity (open circles), and the diagonal line shows the acetonitrile gradient. **b**, The major phosphopeptide from **a** (400 c.p.m.) was subjected to solid-phase sequencing¹², and ^{32}P -radioactivity released after each cycle of Edman degradation is shown. **c**, GSK3- α and GSK3- β were co-immunoprecipitated from the lysates of ^{32}P -labelled cells, denatured in SDS, subjected to SDS-PAGE, transferred to nitrocellulose and autoradiographed¹¹. Lanes 1–3, GSK3 isoforms immunoprecipitated from unstimulated cells; lanes 4–6, GSK3 isoforms immunoprecipitated from insulin-stimulated cells. **d**, GSK3 isoforms from **c** were digested with trypsin, and the resulting phosphopeptides separated by isoelectric focusing¹¹ and identified by autoradiography. Lanes 1 and 4 show the major phosphopeptide resulting from *in vitro* phosphorylation of GSK3- β by PKB and MAPKAP kinase-1, respectively; lanes 2 and 5, the phosphopeptides obtained from GSK3- β and GSK3- α , immunoprecipitated from unstimulated cells; lanes 3 and 6, the phosphopeptides obtained from GSK3- β and GSK3- α , immunoprecipitated from cells stimulated for 5 min with 0.1 μM insulin; the arrow denotes the peptides whose phosphorylation is increased by insulin. The pI values of two markers, Patent Blue (2.4) and azurin (5.7) are indicated.

METHODS. In **a**, PKB- α was immunoprecipitated with the C-terminal antibody from the lysates (0.5 mg protein) of insulin-stimulated L6 myotubes and used to phosphorylate GSK3- β ¹². In **c**, Three 10-cm diameter dishes of L6 myotubes were incubated for 4 h in HEPES-buffered saline¹⁰ containing 50 μM PD 98059, 100 nM rapamycin and 1.5 mCi ml⁻¹ ^{32}P -orthophosphate, stimulated for 5 min with insulin (0.1 μM) or buffer, and GSK3 isoforms co-immunoprecipitated from the lysates as in Fig. 1.

(GSK3- α), was the site of phosphorylation in each phosphopeptide, both *in vitro* (Fig. 4b) and *in vivo* (not shown). The ^{32}P -labelling of other (more acidic) tryptic phosphopeptides was not increased by insulin (Fig. 4d). These peptides have been noted previously in GSK3 from A431 cells and shown to contain phosphoserine and phosphotyrosine¹¹.

PKC- δ , ϵ and ζ are reported to be activated by mitogens, and PKC- ζ activity is stimulated *in vitro* by several inositol phospholipids, including PI(3,4,5)P₃, the product of the PI 3-kinase reaction²⁶. However, purified PKC- ϵ ²⁷, PKC- δ and PKC- ζ (data not shown) all failed to inhibit GSK3- α or GSK3- β *in vitro*. Moreover, although PKC- α , β 1 and γ inhibit GSK3- β *in vitro*²⁷, GSK3- α is unaffected, while their downregulation in L6 myotubes by prolonged incubation with phorbol esters abolishes the activation of MAPKAP kinase-1 in response to subsequent challenge with phorbol esters, but has no effect on the inhibition of GSK3 by insulin (not shown).

Taken together, our results identify GSK3 as the first physiologically relevant substrate for PKB. The stimulation of glycogen synthesis by insulin in skeletal muscle involves the dephosphorylation of Ser residues in glycogen synthase that are phosphorylated by GSK3 *in vitro*². Hence the 40–50% inhibition of GSK3 by insulin, coupled with a similar activation of the relevant glycogen synthase phosphatase²⁸, can account for the stimulation of glycogen synthase by insulin in skeletal muscle² or L6 myotubes²⁹. The activation of glycogen synthase and the resulting stimulation of glycogen synthesis by insulin in L6 myotubes is blocked by wortmannin, but not by PD 98059 (ref. 29), just like the activation of PKB and inhibition of GSK3. However, GSK3 is unlikely to be the only substrate of PKB *in vivo*, and identifying other physiologically relevant substrates will be important because PKB- β is amplified and overexpressed in many ovarian neoplasms²³. □

Identification of the breast cancer susceptibility gene *BRCA2*

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In Western Europe and the United States approximately 1 in 12 women develop breast cancer. A small proportion of breast cancer cases, in particular those arising at a young age, are attributable to a highly penetrant, autosomal dominant predisposition to the disease. The breast cancer susceptibility gene, *BRCA2*, was recently localized to chromosome 13q12-q13. Here we report the identification of a gene in which we have detected six different germline mutations in breast cancer families that are likely to be due to *BRCA2*. Each mutation causes serious disruption to the open reading frame of the transcriptional unit. The results indicate that this is the *BRCA2* gene.

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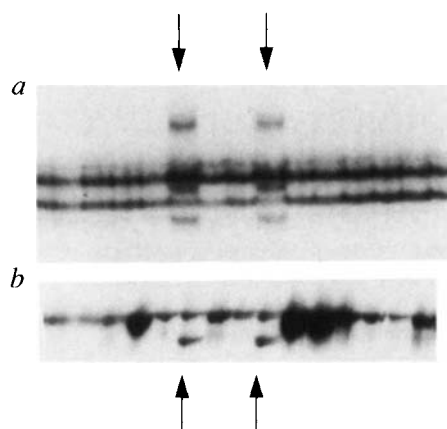


FIG. 1 Detection of the *BRCA2* gene mutation in family IARC 2932. Mutation screening by migration shift assays. The arrows indicate abnormally migrating bands in two early onset breast cancer cases from IARC 2932.

METHODS. A 32 P-labelled, 271-bp genomic fragment was amplified from lymphocyte DNAs from affected individuals in 46 breast cancer families. The PCR product was denatured in 50% formamide and electrophoresed through a, 4.5% non-denaturing polyacrylamide gels and b, 6% denaturing polyacrylamide gels.

Abnormalities of several genes are known to confer susceptibility to breast cancer. The *BRCA1* gene accounts for the large majority of families with both breast and ovarian cancer cases, but only half of families with site-specific breast cancer¹. Using families with multiple cases of early-onset breast cancer showing evidence against linkage to *BRCA1* we recently demonstrated the existence of a second major breast cancer susceptibility locus, *BRCA2*, on chromosome 13q12-q13 (ref. 2). Preliminary studies indicate that mutations in *BRCA2* confer a similar risk of female breast cancer to *BRCA1*. However, the risk of ovarian cancer appears to be lower and the risk of male breast cancer substantially higher. Risks of other cancers, including prostate and laryngeal cancer, may also be elevated in carriers of *BRCA2* mutations (unpublished data).

BRCA2 was originally positioned within a 6-cM region between *D13S289* and *D13S267* that was defined on the basis of meiotic recombinants in early-onset breast cancer cases within clearly linked families². (The genetic map in this region is centromere-*D13S289*-3cM-*D13S260*-1cM-*D13S171*-2cM-*D13S267*-telomere³.) We further mapped the centromeric boundary of the interval within which the gene lies to *D13S260* using a set of Icelandic families (unpublished data). Subsequently, using recombinants in other families and additional microsatellite markers isolated from the region, we established that *BRCA2* is likely to be located in a 600-kb interval centred around *D13S171*. An unexpected contribution to the fine localization of *BRCA2* was provided by the detection of a homozygous somatic deletion in a single pancreatic cancer⁴. The centromeric boundary of this deletion is approximately 300 kb centromeric to *D13S171* and the telomeric boundary close to, but still centromeric of, *D13S171* (ref. 5). Despite the ambiguity of the relationship between this deletion and *BRCA2*, we combined the genetic recombinant information from families and the physical localization from the homozygous deletion, and prioritized analysis of the 300-kb region immediately centromeric to *D13S171*.

Yeast artificial chromosome (YAC)⁶ and P1 artificial chromosome (PAC)⁷ contigs extending approximately 700 kb centromeric and 300 kb telomeric to *D13S171* were constructed and a minimally overlapping set of 14 PACs was identified. Transcribed sequences located on these genomic contigs were identified using two methods: exon amplification (exon trapping) from subcloned PAC DNA⁸, and direct selection by solution hybridization of complementary DNA to PAC genomic DNA⁹. To identify *BRCA2*, genomic DNA fragments of less than 300 bp containing putative coding sequences were screened for mutations. At least one affected member of 46 breast cancer families was examined. Each family included in this set either shows evidence of linkage to *BRCA2*, and/or shows evidence against linkage to *BRCA1*, and/or has not been found to carry a *BRCA1* mutation, and/or includes a case of male breast cancer. Most, but probably not all, of these families would be expected to have cases caused by *BRCA2* mutations.

Disease-associated mutations in most known cancer susceptibility genes usually result in truncation of the encoded protein and inactivation of critical functions. In the course of the mutational screen of candidate coding sequences from the *BRCA2* region, the first detected sequence variant that was predicted to disrupt translation of an encoded protein was observed in IARC 2932 (Fig. 1). This family is clearly linked to *BRCA2* with a multipoint LOD score of 3.01 using *D13S260* and *D13S267*. A deletion of 6 bp removes the last five bases of the exon examined (exon S66), deletes the conserved G of the 5' splice site of the intron, and directly converts the codon TTT for phenylalanine to the termination codon TAA. By sequencing, this mutation has been detected in lymphocyte DNA from two other early-onset breast cancer cases in this family. The individuals examined share only the disease-associated haplotype. The mutation is absent in more than 500 chromosomes from normal individuals and in the remaining families and cancers. This finding therefore identified a strong candidate for the *BRCA2* gene.

TABLE 1 *BRCA2* mutations in breast cancer families

	FBCs	FBCs < 50	OvCs	MBCs	LOD score at <i>BRCA1</i>	LOD score at <i>BRCA2</i>	<i>BRCA2</i> mutation
IARC 2932	15	10	0	0	-2.38	3.01	CCC.TTT.CGgtaa
IARC 3594	6	5	0	0	nd	nd	CAT.AAC.TCT.CTA
CRC B211	5	3	4	0	-0.48	0.49	AGT.CTT.CAC
CRC B196	17	12	0	0	-2.21	0.92	AAA.ACT.GAA.ACT
Montreal 681	3	2	0	1	nd	nd	GCA.AGT.GGA
Montreal 440	2	2	0	2	nd	nd	GAT.AAA.CAA.GCA

LOD scores at *BRCA1* were calculated using the markers *D17S250* and *D17S579*; those at *BRCA2* were calculated using the markers *D13S260* and *D13S267*. Exon sequence is denoted by upper case, intron sequence by lower case; Codons are indicated by stops. The underlined letters indicate the deleted bases in each family. Abbreviations: FBCs, female breast cancers; OvCs, ovarian cancers, MBCs, male breast cancers.

FIG. 2 Predicted amino acid sequence of the *BRCA2* gene. The positions of the frameshift mutations indicated in Table 1 are boxed, and the positions of intron-exon boundaries are arrowed above the amino acid sequence.

METHODS. Exon S66 and others that had been trapped in association with it were used to isolate segments of the candidate cDNA by hybridization to normal human fetal brain, placental, monocyte and breast cancer cDNA libraries. Additional fragments were isolated by PCR amplification from known exon sequences to vector ends. In the course of these analyses, other previously trapped exons and cDNAs selected by solution hybridization were incorporated into an extended cDNA sequence. In addition, the exon prediction program Genemark was used to define the location of adjacent candidate transcribed sequences from the genomic sequence. Putative intron-exon boundaries were confirmed by amplification from cDNA and direct sequencing of amplification products. Northern analysis indicates that the transcript from the *BRCA2* gene is large (approximately 10–12 kb), and hence the N terminus of the *BRCA2* protein may well be missing from the above sequence.

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HIGKSMPNVLEDEVYETVVDTSSEDSFSLCFSKCRCTKNLQKVRTSKTRKKIFHEANADEC 60
EKSKNQVKEKYSFVSEVEPNDDPLDSNVANQKPFESGSDKISKEVVP SLACEWSQLTSL 120
GLNGAQMEKIPLLHISSCDQNISEKDLLDTENKRRKDFLTSENSLPRISLLPKSEKPLNE 180
ETVVNKRDEEQHLESHTDCILAVKQAISGTSVPASSPQGIKKSIFRIRESKPETFNASFS 240
GHMTDPNFKKETEASESGLEIHTVCSQKEDSLCPNLIDNGSWPATTTQNSVALKNAGLIS 300
TLKKKTNKFIIYAIHDETSYKGGKIPKDKQKSELINCSAQFEANAFEAPLTFANADSGLLHS 360
SVKRSQNDSEPTLSLTSSFGTILRKCSRNETCSNNTVISQDLDYKEAKCNKEKLQLF 420
ITPEADSLSCLQEGQCENDPKSKKVSDIKEEVLAACHPVQHSKVEYSDTDFQSQKSLLY 480
DHENASTLILTPTSKDVLNLMISRGKESYKMSDKLKGNNYESDVELTKNIPMEKNQDV 540
CALNENYKNVELLPPEKYMVASPSRQVQFNQNTNLRVIQKNQEETTSISKITVNPDSSE 600
LFSNENNFVVFQVANERNNLALGNTKELHETDLTCVNEPIFKNSTMVLYGDTGKQATQV 660
SIKKDLVYVLAENKNSVKQHIKMTLGQDLKSDISLNDIKIPEKNNDYMNKAWAGLGPIS 720
NHSFGGSPRTASKEIKLSEHNKSKMFFKDIEEQYPTSLACVEIVNTLALDNQKLSK 780
PQSINTVSAHLQSSVVVSDCKNSHITPQMLFSKQDFNSNHNLTSPQKEITELSTILED 840
GSQFEFTQFRKPSYILQKSTFEVPEQMILTKTSEECDADLHVIMNAPSIGQVDSSKQ 900
FEGTVEIKRKFAGLLKNDCKNSASGYLTDENEVGRFGFYSAHGTKLVNSTEALQKAVKLF 960
SDIENISEETSAEVHPISSLSSKCHDSVVSMPKIEHNNDKTVSEKNKCKLILQNNIEMT 1020
TGTFFVEITENYKRNTEENEDNKYTAASRNHNLEFDGSDSKNDTVCIHKDETDLLFTDQ 1080
HNICLLSGQFMKEGNTQIKEDLSLTFLEVAKAQEACHGNTSNKEQLTATKTEQNIKF 1140
ETSDTFFQTASGKNISVAKESFNKIVNFFDQKPEELHNFSLNSELHSDIRKNKMDLSYE 1200
ETDIVKHKILKESVPVGTGNLQVTFQGGPERDEKIKEPTLLGFHTAGSKVKVIAKESLDK 1260
VKNLDFEKEQGTSEITFSHGWAKTLKYREACKDLELACETIETIAPKCKEMQNSLNND 1320
KNLVSIIETVVPKLLSDNLRCQTENLKTSSKIFLKVKHVENVEKETAKSPATCYTNQSPY 1380
SVIENSALAFYTSCSRKTSVSTSLLEAKKWLRREGIFDGGQPERINTADYVGNLYENNSN 1440
STIAENDKNHLEKQDLYLSSSSMSNSYSYHSDEVYNDSDGYLSKNKLDGSGIEPVLKNVED 1500
QKNTSFSKVISNVK DANAYPTQVNEDICVEELVTSSSPCKNNAIKLSISNSNNEVGP 1560
PAFRIASGKIVCVSHETIKKVKDIFTDSFSKVIKENNENKSKICQTKIMAGCYEALDDSE 1620
DILHNSLDNDECSHSHKVFADIQSEILQHNQMSGLEKVSISPCDVSLETSIDICKCS 1680
IGKLHKSVSANTCGIFSTASGKSVQVSDASLQNAQVVFSEIEDSTKQVPSKVLFSKNEH 1740
SDQLTREENTAIRTEHLISQKGFYSNVVNSSAFSGFSTASGKQVSILESSLHKVKGVL 1800
EFDLIRTEHSYHSPTSRQNVSKILPRVDKRNPEHCNVSEMEKTCSEFKLSNNLNVEGG 1860
SSENNHSIKVSPYLSQFQDQKQLVLGTVSLVENIHVLGKEQASPKNVKMEIGKTEFTFS 1920
DVPVKTNIIEVCSTYSKDSYFETEAVEIAKAFMEDDELTDKSLPASHLSLFTCPENEE 1980
MVLNSNRIGKRRGEPLILVGEPSIKRNLNNEFDRIENQESKLSKASTPDGTIKDRRLF 2040
VHHVLEPITCVFPTTKERQEIQNPFTAPGQEFLSKSHLYEHLTEKSSSNLAVSGHP 2100
FYQVSGNKNGKMRKLITGRPTKVFVPPFKTKSHFHRVEQCVRNINLEGNRQKQIDGHG 2160
SDDSKNKINDNEIHQFNKNNNSQAAAVTFTKCEEPDLITSLQNAQIDQDMRIKKKQK 2220
RVFPQPGSLYLAKTSTLPRISLKAAGVGQVPSACHKQLYTYGVSKHCIKINSKNAESFQ 2280
FHTEDYFGKESLWTGKGIQLADGGWLIPSDNGKAGKEEFYRAKCDVKAT 2329
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To characterize this gene further, exon S66 was used to isolate a series of cDNA clones which represented segments of the *BRCA2* candidate (see Fig. 2 legend). At this stage the initial shotgun sequence data from a 900-kb region thought to contain *BRCA2* was completed at the Sanger Centre and Washington University and became available to us through the public release of the assembled sequence (at <ftp://ftp.sanger.ac.uk/pub/human/sequences/13q> and <ftp://genome.wustl.edu/pub/gsc1/bca2> from 23 November 1995). From alignment of the cDNA and genomic sequence data, the candidate *BRCA2* gene was found to lie in three sequence contigs which also contained other previously isolated transcribed sequences. The exon and open reading frame prediction program Genemark was used to define putative additional 5' exons of the gene. Contiguity of the transcription unit was confirmed by reverse-transcription-polymerase chain reaction (RT-PCR) on cDNA and sequence analysis. The availability of extensive sequence information at the cDNA and genomic level allowed mutational analysis of further coding regions of the putative *BRCA2* gene in samples from breast cancer families.

A TG deletion and a TT deletion were detected in families CRC B196 and CRC B211 respectively (Table 1). In both famil-

ies the mutation has been detected by sequencing other individuals with early onset breast cancer who share only the haplotype of 13q microsatellite markers that segregates with the disease. Therefore, the mutations are on the disease-associated chromosomes. A CT deletion was detected in family IARC 3594. This mutation has arisen within a short repetitive sequence (CTCTCT), a feature that is characteristic of deletion/insertion mutations in many genes, and which is presumed to be due to slippage during DNA synthesis. Finally, a T deletion and an AAAC deletion have been found in Montreal 681 and 440, respectively. Both these families include a male breast cancer case, and previous analyses have indicated that the large majority of such families will have *BRCA2* mutations¹⁰. All these mutations are predicted to generate frameshifts leading to premature termination codons. None of the mutations have been found in over 500 chromosomes from healthy women and are therefore unlikely to be polymorphisms. The identification of several different germline mutations that truncate the encoded protein in breast cancer families that are highly likely to be due to *BRCA2* strongly suggests that we have identified the *BRCA2* gene.

Northern analysis has demonstrated that *BRCA2* is encoded by a transcript of 10–12 kb (data not shown), which is present

in normal breast epithelial cells, placenta and the breast cancer cell line MCF7. This suggests that our present contig of cDNAs covering approximately 7.3 kb (including 300 bp of 3' untranslated sequence) may not include the whole *BRCA2* coding sequence. The known sequence of 2,329 amino acids encoded by the *BRCA2* gene does not show strong homology to sequences in the publicly available DNA or protein databases, and therefore we have no clues to its functions. However, some weak matches were detected including, intriguingly, a very weak similarity to the *BRCA1* protein over a restricted region (amino acids 1394–1474 in *BRCA1*, and 1783–1863 in the portion of *BRCA2* shown in Fig. 2). The significance of this is unclear.

Loss of heterozygosity on chromosome 13q has been observed in sporadic breast and other cancers, suggesting that there is a somatically mutated tumour suppressor gene in this region^{11–13}. *BRCA2* is a strong candidate for this gene, and the analysis of a large series of cancers is underway to investigate if *BRCA2* is somatically mutated during oncogenesis.

The identification of *BRCA2* should now allow more comprehensive evaluation of families at high risk of developing breast cancer. However, the roles of environmental, lifestyle or genetic factors in modifying the risks of cancer in gene carriers are unknown, and further studies will be required before routine diagnosis of carrier status can be considered. □

M. Ponder, H. Vasen, J. Feunteun, O. Serova, R. Gwilliam, S. Humphray, M. Leversha, Y. Ramsey, H. King, D. Cain, D. Averill, P. Mitchell, M. Crompton, C. Cochran, J. Marks, D. Iglehart, R. Wiseman, J. Lancaster and the members of the Human Sequencing teams at the Sanger Centre and the Genome Sequencing Centre at Washington University for making their data publicly available; M. Schutte and S. Kern for discussions; and C. Marshall, C. Cooper and P. Garland for encouragement. This work was supported by the Cancer Research Campaign, BREAKTHROUGH Breast Cancer Charity and the Jean Rook Appeal, the Institute of Cancer Research, the Wellcome Trust, the Medical Research Council, the Imperial Cancer Research Fund, the U.S. Army, Duke University SPORE in Breast Cancer, the National Cancer Institute, the Icelandic Cancer Research Fund, the Nordic Cancer Union, the Ligue National contre le Cancer, the Dutch Cancer Society, the Cancer Research Society, the Canadian Breast Cancer Foundation, the Fonds de la recherche en Santé du Québec, and the Canadian Genetic Diseases Network. Access to *BRCA2* sequences can be obtained from rich_w@icr.ac.uk.

RETRACTION

Cloning and functional expression of a rat heart K_{ATP} channel

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In this letter we described the cloning and expression of an inward rectifier potassium-channel subunit from rat heart (Kir 3.4) which, when transfected into HEK293 and BHK21 cells, endowed them with ATP-sensitive potassium channels. Since this paper appeared, we have not been able regularly to reproduce those findings. In addition, the data presented by Krapivinsky *et al.*¹ presents a compelling argument that Kir 3.4 is an intrinsic component of the channel underlying I_{KACH} in atrium, and that it does not contribute to the channel underlying cardiac I_{KATP} . Therefore, we cannot support our previous statement that Kir 3.4 represents a subunit of cardiac K_{ATP} channels. □

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